TECHNICAL MANUAL

PowerPlex® Fusion 6C System

Instructions for Use of Products
DC2705 and DC2720

1/15
TMD045

PowerPlex® Fusion 6C System

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: genetic@promega.com

1. Description

STR (short tandem repeat) loci consist of short, repetitive sequence elements 3–7 base pairs in length (1–4). These repeats are well distributed throughout the human genome and are a rich source of highly polymorphic markers, which may be detected using the polymerase chain reaction (5–9). Alleles of STR loci are differentiated by the number of copies of the repeat sequence contained within the amplified region and are distinguished from one another using fluorescence detection following electrophoretic separation.

The PowerPlex® Fusion 6C System[a–h] is a 27-locus multiplex for human identification applications including forensic analysis, relationship testing and research use. This six-color system allows co-amplification and fluorescent detection of the 18 autosomal loci in the expanded CODIS core loci (CSF1PO, FGA, TH01, vWA, D1S1656, D2S1338, D2S441, D3S1358, D5S818, D7S820, D8S1179, D10S1248, D12S391, D13S317, D16S539, D18S51, D19S433 and D21S11) as well as Amelogenin and DYS391 for gender determination. The Penta D, Penta E, D22S1045, TPOX and SE33 loci are also included to increase discrimination and allow searching of databases that include profiles with these loci. Finally, two rapidly mutating Y-STR loci, DYS570 and DYS576, are included in the multiplex. This extended panel of STR markers is intended to satisfy both CODIS and ESS recommendations.

The PowerPlex® Fusion 6C System is compatible with the Applied Biosystems® 3500 and 3500xL Genetic Analyzers as well as Applied Biosystems® 3130 and 3130*xl* Genetic Analyzers with Data Collection Software Version 4.0 with the DC v4 6-Dye Module v1 License (Life Technologies). Amplification and detection instrumentation may vary. You may need to optimize protocols including amount of template DNA, cycle number and injection conditions for your laboratory instrumentation. In-house validation should be performed.

The PowerPlex® Fusion 6C System provides all materials necessary to amplify STR regions of human genomic DNA, including a hot-start thermostable DNA polymerase, which is a component of the PowerPlex® Fusion 6C 5X Master Mix. This manual contains protocols for use of the PowerPlex® Fusion 6C System with the GeneAmp® PCR System

9700 thermal cycler and Veriti® 96-Well Thermal Cycler in addition to protocols to separate amplified products and detect separated material (Figure 1). A protocol to operate the fluorescence-detection instrument should be obtained from the instrument manufacturer. The 5X AmpSolution Reagent (Cat.# DM1231) is required for direct amplification of DNA from storage card punches in a 12.5µl reaction with the PowerPlex® Fusion 6C System.

Information about other Promega fluorescent STR systems is available upon request from Promega or online at: **www.promega.com**

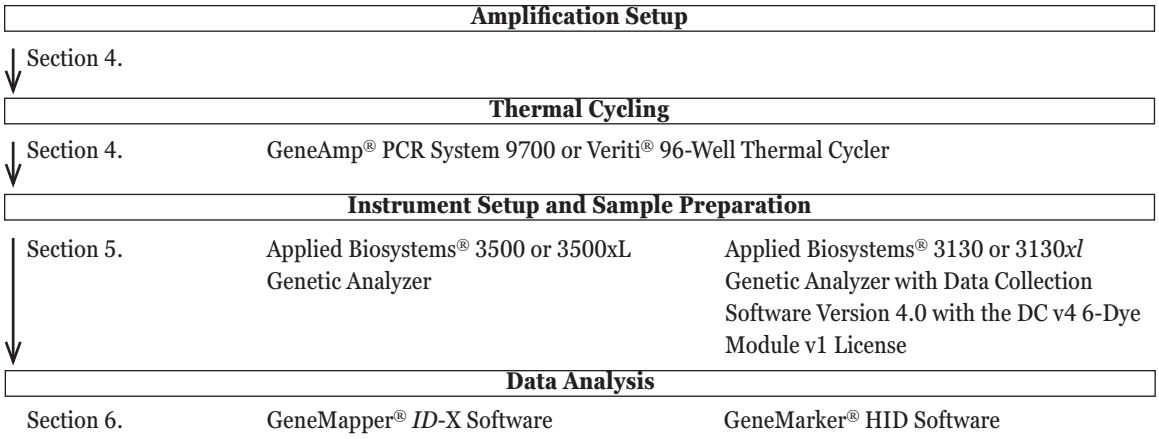

Figure 1. An overview of the PowerPlex® Fusion 6C System protocol.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
PowerPlex® Fusion 6C System	**50 (or 100 direct-amp) reactions**	**DC2705**

Not For Medical Diagnostic Use. This system contains sufficient reagents for 50 reactions of 25µl each or 100 direct-amplification reactions of 12.5µl each. Includes:

Pre-amplification Components Box

- 250µl PowerPlex® Fusion 6C 5X Master Mix
- 250µl PowerPlex® Fusion 6C 5X Primer Pair Mix
- 25µl 2800M Control DNA, 10ng/µl
- 1,250µl Water, Amplification Grade

Post-amplification Components Box

- 25µl PowerPlex® Fusion 6C Allelic Ladder Mix
- 200µl WEN Internal Lane Standard 500

2. Product Components and Storage Conditions (continued)

PRODUCT	SIZE	CAT.#
PowerPlex® Fusion 6C System	**200 (or 400 direct-amp) reactions**	**DC2720**

Not For Medical Diagnostic Use. This system contains sufficient reagents for 200 reactions of 25µl each or 400 direct-amplification reactions of 12.5µl each. Includes:

Pre-amplification Components Box

- 4 × 250µl PowerPlex® Fusion 6C 5X Master Mix
- 4 × 250µl PowerPlex® Fusion 6C 5X Primer Pair Mix
- 25µl 2800M Control DNA, 10ng/µl
- 5 × 1,250µl Water, Amplification Grade

Post-amplification Components Box

- 4 × 25µl PowerPlex® Fusion 6C Allelic Ladder Mix
- 2 × 200µl WEN Internal Lane Standard 500

The PowerPlex® Fusion 6C Allelic Ladder Mix is provided in a separate, sealed bag for shipping. This component should be moved to the post-amplification box after opening. For the 200-reaction PowerPlex® Fusion 6C System (Cat.# DC2720), the Water, Amplification Grade, is provided in a separate, sealed bag for shipping. Store this component with the pre-amplification components after opening.

Storage Conditions: Upon receipt, store all components at –30°C to –10°C in a nonfrost-free freezer. Make sure that the 2800M Control DNA is stored at 2–10°C for at least 24 hours before use. After the first use, store the PowerPlex® Fusion 6C System components at 2–10°C, where they are stable for 6 months. Do not refreeze. The PowerPlex® Fusion 6C 5X Primer Pair Mix, PowerPlex® Fusion 6C Allelic Ladder Mix and WEN Internal Lane Standard 500 (WEN ILS 500) are light-sensitive and must be stored in the dark. We strongly recommend that pre-amplification and post-amplification reagents be stored and used separately with different pipettes, tube racks, etc.

Available Separately

PRODUCT	SIZE	CAT.#
PunchSolution™ Kit	**100 preps**	**DC9271**
SwabSolution™ Kit	**100 preps**	**DC8271**
5X AmpSolution™ Reagent	**100 preps**	**DM1231**

The PunchSolution™ Kit is required to process nonFTA punches prior to direct amplification. The SwabSolution™ Kit is required to process swabs prior to direct amplification. The 5X AmpSolution Reagent is required for direct amplification of DNA from storage card punches in a 12.5µl reaction volume. Both the PunchSolution™ Kit and SwabSolution™ Kit include the 5X AmpSolution™ Reagent.

The proper panels, bins and stutter text files for use with GeneMapper® *ID*-X software are available for download at: **www.promega.com/resources/tools/genemapper-id-software-panels-and-bin-sets/**

Matrix standards are required for initial setup of the color separation matrix. The PowerPlex® 6C Matrix Standard is provided separately and is compatible with the Applied Biosystems® 3500 and 3500xL Genetic Analyzers and Applied Biosystems® 3130 and 3130*xl* Genetic Analyzers with Data Collection Software Version 4.0 and DC v4 6-Dye Module v1 License (PowerPlex® 6C Matrix Standard, Cat.# DG4900).

3. Before You Begin

3.A. Precautions

The application of PCR-based typing for forensic or paternity casework requires validation studies and quality-control measures that are not contained in this manual (10,11). Guidelines for the validation process are published in the *Internal Validation of STR Systems Reference Manual* (12).

The quality of purified DNA or direct-amplification samples, small changes in buffers, ionic strength, primer concentrations, reaction volume, choice of thermal cycler and thermal cycling conditions can affect PCR success. We suggest strict adherence to recommended procedures for amplification and fluorescence detection. Additional research and validation are required if any modifications to the recommended protocols are made.

PCR-based STR analysis is subject to contamination by very small amounts of human DNA. Extreme care should be taken to avoid cross-contamination when preparing template DNA, handling primer pairs, assembling amplification reactions and analyzing amplification products. Reagents and materials used prior to amplification (PowerPlex® Fusion 6C 5X Master Mix, PowerPlex® Fusion 6C 5X Primer Pair Mix, 2800M Control DNA and Water, Amplification Grade) are provided in a separate box and should be stored separately from those used following amplification (PowerPlex® Fusion 6C Allelic Ladder Mix and WEN Internal Lane Standard 500). Always include a negative control reaction (i.e., no template) to detect reagent contamination. We highly recommend the use of gloves and aerosol-resistant pipette tips.

Some reagents used in the analysis of STR products are potentially hazardous and should be handled accordingly. Formamide is an irritant and a teratogen; avoid inhalation and contact with skin. Read the warning label, and take appropriate precautions when handling this substance. Always wear gloves and safety glasses when working with formamide.

3.B. Spectral Calibration

Proper spectral calibration is critical to evaluate multicolor systems with the Applied Biosystems® 3500 and 3500xL Genetic Analyzers and Applied Biosystems® 3130 and 3130*xl* Genetic Analyzers with Data Collection Software Version 4.0 and DC v4 6-Dye Module v1 License. A matrix must be generated for each individual instrument.

For protocols and additional information on spectral calibration on these instruments, see the *PowerPlex® 6C Matrix Standard Technical Manual* #TMD046. This manual is available at: **www.promega.com/protocols/**

4. Protocols for DNA Amplification Using the PowerPlex® Fusion 6C System

The PowerPlex® Fusion 6C System was developed for amplification of extracted DNA and direct-amplification samples. Slight protocol variations are recommended for optimal performance with each template source. Protocols for amplification using extracted DNA (Section 4.A), FTA® and nonFTA storage card punches (Section 4.B) and swabs (Section 4.C) are included in the following amplification sections.

The PowerPlex® Fusion 6C System is compatible with the GeneAmp® PCR System 9700 thermal cycler with a silver-plated or gold-plated sample block and the Veriti® 96-Well Thermal Cycler. This system has not been tested with the Veriti® 96-Well Fast Thermal Cycler or GeneAmp® PCR System 9700 with an aluminum block.

The use of gloves and aerosol-resistant pipette tips is highly recommended to prevent cross-contamination. Keep all pre-amplification and post-amplification reagents in separate rooms. Prepare amplification reactions in a room dedicated for reaction setup. Use equipment and supplies dedicated for amplification setup.

Meticulous care must be taken to ensure successful amplification. A guide to amplification troubleshooting is provided in Section 9.

The concentration of 2800M Control DNA was determined by measuring absorbance at 260nm. Quantification of this control DNA by other methods, such as qPCR, may result in a different value. Prepare a fresh DNA dilution for each set of amplifications. Do not store diluted DNA (e.g., 0.25ng/µl or less).

4.A. Amplification of Extracted DNA in a 25µl Reaction Volume

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700, 96-Well, with a gold-plated or silver-plated sample block or Veriti® 96-Well Thermal Cycler (Applied Biosystems)
- centrifuge compatible with a 96-well plate
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips

We routinely amplify 1.0ng of template DNA in a 25µl reaction volume using the protocol detailed below.

Amplification Setup

1. At the first use, thaw the PowerPlex® Fusion 6C 5X Master Mix, PowerPlex® Fusion 6C 5X Primer Pair Mix and Water, Amplification Grade, completely. After the first use, store the reagents at 2–10°C.

   Note: Centrifuge tubes briefly to bring contents to the bottom, then vortex reagents for 15 seconds before each use. Do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.

3. Use a clean MicroAmp® plate for reaction assembly, and label appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label appropriately.

4. Add the final volume of each reagent listed in Table 1 to a sterile tube.

Table 1. PCR Amplification Mix for Amplification of Extracted DNA.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 25.0µl	×		=	
PowerPlex® Fusion 6C 5X Master Mix	5.0µl	×		=	
PowerPlex® Fusion 6C 5X Primer Pair Mix	5.0µl	×		=	
template DNA (1.0ng)[2,3]	up to 15µl				
total reaction volume	25µl				

[1]Add Water, Amplification Grade, to the tube first, then add PowerPlex® Fusion 6C 5X Master Mix and PowerPlex® Fusion 6C 5X Primer Pair Mix. The template DNA will be added at Step 6.

[2]Store DNA templates in TE^{-4} buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA) or TE^{-4} buffer with 20µg/ml glycogen. If the DNA template is stored in TE buffer that is not pH 8.0 or contains a higher EDTA concentration, the volume of DNA added should not exceed 20% of the final reaction volume. PCR amplification efficiency and quality can be greatly altered by changes in pH (due to added Tris-HCl), available magnesium concentration (due to chelation by EDTA) or other PCR inhibitors, which may be present at low concentrations depending on the source of the template DNA and the extraction procedure used.

[3]Apparent DNA concentrations can differ, depending on the DNA quantification method used (13). The amount of DNA template recommended here is based on DNA concentrations determined by measuring absorbance at 260nm. We strongly recommend that you perform experiments to determine the optimal DNA amount based on your DNA quantification method.

5. Vortex the PCR amplification mix for 5–10 seconds, then pipet PCR amplification mix into each reaction well.

! Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or locus-to-locus imbalance.

6. Add the template DNA (1.0ng) for each sample to the respective well containing PCR amplification mix.

   Note: The PowerPlex® Fusion 6C System was optimized and balanced using 1.0ng of DNA template. The amount of DNA template used in your laboratory should be based on the results of your internal validation and may be different.

7. For the positive amplification control, vortex the tube of 2800M Control DNA, then dilute an aliquot to 1.0ng in the desired template DNA volume. Add 1.0ng of diluted DNA to a reaction well containing PCR amplification mix.

8. For the negative amplification control, pipet Water, Amplification Grade, or TE^{-4} buffer instead of template DNA into a reaction well containing PCR amplification mix.

9. Seal or cap the plate, or close the tubes. **Optional:** Briefly centrifuge the plate to bring contents to the bottom of the wells and remove any air bubbles.

4.A. Amplification of Extracted DNA in a 25µl Reaction Volume (continued)

Thermal Cycling

Amplification and detection instrumentation may vary. You may need to optimize protocols including the amount of template DNA, cycle number, injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 29 cycles works well for 1.0ng of purified DNA templates.

1. Place the MicroAmp® plate or reaction tubes in the thermal cycler.
2. Select and run the recommended protocol, which is provided below and in Figure 2. The total cycling time is approximately 1 hour.

   Notes:

   1. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.
   2. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a silver-plated or gold-plated sample block. The ramp speed is set after the thermal cycling run is started. When the Select Method Options screen appears, select "Max" for the ramp speed and enter the reaction volume.

Thermal Cycling Protocol
96°C for 1 minute, then:
96°C for 5 seconds 60°C for 1 minute for 29 cycles, then:
60°C for 10 minutes
4°C soak

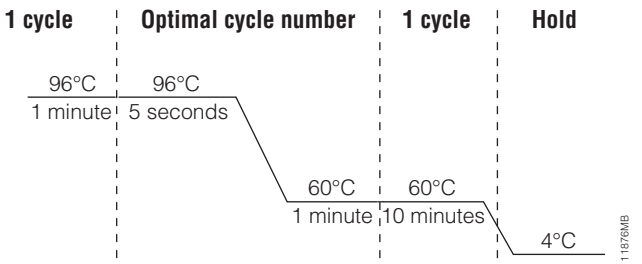

Figure 2. The thermal cycling protocol for the GeneAmp® PCR System 9700 and Veriti® 96-Well Thermal Cycler.

3. After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at –20°C in a light-protected box.

   Note: Long-term storage of amplified samples at 4°C or higher may produce artifacts.

4.B. Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700, 96-Well, with a gold-plated or silver-plated sample block or Veriti® 96-Well Thermal Cycler (Applied Biosystems)
- centrifuge compatible with a 96-well plate
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- PunchSolution™ Kit (Cat.# DC9271) for nonFTA card punches
- 5X AmpSolution™ Reagent for FTA® card punches (Cat.# DM1231)
- 1.2mm Harris Micro-Punch or equivalent manual punch and cutting mat or automated punch system

This section contains a protocol for direct amplification of DNA from storage card punches in 12.5µl reaction volumes using the PowerPlex® Fusion 6C System and GeneAmp® PCR System 9700 or Veriti® 96-Well Thermal Cycler. A protocol for direct amplification of DNA from storage card punches in 25µl reaction volumes is provided in Section 11.D.

For 12.5µl amplification reactions, we recommend amplifying one 1.2mm punch of an FTA® or nonFTA storage card containing buccal cells or whole blood.

FTA®-based sample types include:

- Buccal cells collected on FTA® cards with Whatman EasiCollect™ or Fitzco Sampact™ devices
- Buccal cells collected with swabs transferred to FTA® or Indicating FTA® cards
- Liquid blood (from collection or storage Vacutainer® tubes or finger sticks) spotted onto FTA® cards

NonFTA sample types include:

- Buccal samples on Bode Buccal DNA Collector™ devices
- Blood and buccal samples on nonFTA cards (e.g., S&S 903)

Pretreat nonFTA sample types with the PunchSolution™ Kit (Cat.# DC9271) to lyse nonFTA samples before adding the PCR amplification mix. For more information, see the *PunchSolution™ Kit Technical Manual* #TMD038. Failure to pretreat these samples may result in incomplete profiles.

Use a manual punch tool with a 1.2mm tip to manually create sample disks from a storage card. Place tip near the center of the sample spot, and with a twisting or pressing action, cut a 1.2mm sample disk. Use the plunger to eject the disk into the appropriate well of a reaction plate.

Automated punchers also can be used to create sample disks. Refer to the user's guide for your instrument for assistance with generating 1.2mm disks, technical advice and troubleshooting information.

Note: Static may be problematic when adding a punch to a well. For FTA® card punches, adding PCR amplification mix to the well before adding the punch may help alleviate static problems. For nonFTA card punches, adding PunchSolution™ Reagent to the well before adding the punch during pretreatment may help alleviate static problems.

4.B. Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume (continued)

Amplification Setup

1. At the first use, thaw the PowerPlex® Fusion 6C 5X Master Mix, PowerPlex® Fusion 6C 5X Primer Pair Mix and Water, Amplification Grade, completely. After the first use, store the reagents at 2–10°C.

   Note: Centrifuge tubes briefly to bring contents to the bottom, then vortex reagents for 15 seconds before each use. Do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.

3. Use a clean MicroAmp® plate for reaction assembly, and label appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label appropriately.

4. Add the final volume of each reagent listed in Table 2 to a sterile tube.

Table 2. PCR Amplification Mix for Direct Amplification of DNA from Storage Card Punches Using a 12.5µl Reaction Volume.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	5.0µl	×		=	
PowerPlex® Fusion 6C 5X Master Mix	2.5µl	×		=	
PowerPlex® Fusion 6C 5X Primer Pair Mix	2.5µl	×		=	
5X AmpSolution™ Reagent	2.5µl	×		=	
total reaction volume	12.5µl				

[1]Add Water, Amplification Grade, to the tube first, then add PowerPlex® Fusion 6C 5X Master Mix, PowerPlex® Fusion 6C 5X Primer Pair Mix and 5X AmpSolution™ Reagent. For FTA® card punches, the template DNA will be added at Step 6.

5. Vortex the PCR amplification mix for 5–10 seconds, then pipet 12.5µl of PCR amplification mix into each reaction well.

! Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or locus-to-locus imbalance.

6. For FTA® storage cards, add one 1.2mm punch from a card containing buccal cells or whole blood to the appropriate wells of the reaction plate. For nonFTA card punches, add the PCR amplification mix to the well or tube containing the PunchSolution™ Reagent-treated punch.

   Note: It also is acceptable to add the FTA® card punch first, then add the PCR amplification mix.

7. For the positive amplification control, vortex the tube of 2800M Control DNA, then add 1µl (10ng) to a reaction well containing 12.5µl of PCR amplification mix.

   Notes:

   1. Optimization of the amount of control DNA may be required, depending on cycling conditions and laboratory preferences.
   2. When performing more than 25 cycles with 12.5µl volume reactions, you may need to dilute the 2800M Control DNA to 5ng/µl prior to adding 1µl (5ng) to positive control reactions.
   3. Do not include blank storage card punches in the positive control reactions.

8. Reserve a well containing PCR amplification mix as a negative amplification control.

   Note: An additional negative control with a blank punch may be performed to detect contamination from the storage card or punch device.

9. Seal or cap the plate, or close the tubes. Briefly centrifuge reactions to bring storage card punches to the bottom of the wells and remove air bubbles.

Thermal Cycling

Amplification and detection instrumentation may vary. You will need to optimize protocols including the number of storage card punches, cycle number (23–26 cycles), injection time and loading volume for your laboratory instrumentation. Testing at Promega shows that 25 cycles works well for a variety of sample types. Buccal samples may require more amplification cycles than blood samples. NonFTA card punches may require fewer amplification cycles than FTA® punches. Cycle number should be optimized in each laboratory for each sample type.

1. Place the MicroAmp® plate or reaction tubes in the thermal cycler.
2. Select and run the recommended protocol, which is provided below and in Figure 3. The total cycling time is approximately 1 hour.

   Notes:

   1. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.
   2. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a silver-plated or gold-plated sample block. The ramp speed is set after the thermal cycling run is started. When the Select Method Options screen appears, select "Max" for the ramp speed and enter the reaction volume.

Thermal Cycling Protocol
96°C for 1 minute, then:
96°C for 5 seconds 60°C for 1 minute for 25 cycles, then:
60°C for 10 minutes
4°C soak

4.B. Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume (continued)

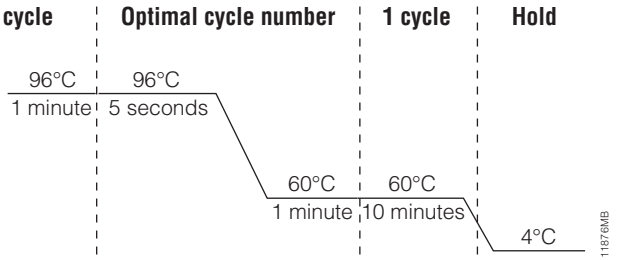

Figure 3. Thermal cycling protocol for the GeneAmp® PCR System 9700 and Veriti® 96-Well Thermal Cycler.

3. After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at –20°C in a light-protected box.

   Note: Long-term storage of amplified samples at 4°C or higher may produce artifacts.

PCR Optimization

Cycle number should be optimized based on the results of an initial experiment to determine the sensitivity with your collection method, sample types and instrumentation.

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.
2. Place one 1.2mm storage card punch containing buccal cells or whole blood in each well of a reaction plate. Be sure to pretreat nonFTA samples with the PunchSolution™ Kit (Cat.# DC9271).
3. Prepare four identical reaction plates with punches from the same samples.
4. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (23–26 cycles).
5. Following amplification, use your laboratory's validated separation and detection protocols to determine the optimal cycle number for the sample type.

4.C. Direct Amplification of DNA from Swabs in a 12.5µl Reaction Volume

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700, 96-Well, with a gold-plated or silver-plated sample block or Veriti® 96-Well Thermal Cycler (Applied Biosystems)
- centrifuge compatible with a 96-well plate
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- SwabSolution™ Kit (Cat.# DC8271)

This section contains a protocol for amplifying DNA from swab extracts in 12.5µl reaction volumes using the PowerPlex® Fusion 6C System and GeneAmp® PCR System 9700 or Veriti® 96-Well Thermal Cycler. A protocol for direct amplification of DNA from swabs in 25µl reaction volumes is provided in Section 11.E.

Pretreat OmniSwab™ (GE Healthcare) or cotton swabs with the SwabSolution™ Kit (Cat.# DC8271) as described in the *SwabSolution™ Kit Technical Manual* #TMD037 to generate a swab extract.

Amplification Setup

1. At the first use, thaw the PowerPlex® Fusion 6C 5X Master Mix, PowerPlex® Fusion 6C 5X Primer Pair Mix and Water, Amplification Grade, completely. After the first use, store the reagents at 2–10°C.

   Note: Centrifuge tubes briefly to bring contents to the bottom, then vortex reagents for 15 seconds before each use. Do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.

3. Use a clean MicroAmp® plate for reaction assembly, and label appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label appropriately.

4. Add the final volume of each reagent listed in Table 3 to a sterile tube.

Table 3. PCR Amplification Mix for Direct Amplification of DNA from Swabs Using a 12.5µl Reaction Volume.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	5.5µl	×		=	
PowerPlex® Fusion 6C 5X Master Mix	2.5µl	×		=	
PowerPlex® Fusion 6C 5X Primer Pair Mix	2.5µl	×		=	
swab extract	2.0µl				
total reaction volume	12.5µl				

[1]Add Water, Amplification Grade, to the tube first, then add PowerPlex® Fusion 6C 5X Master Mix and PowerPlex® Fusion 6C 5X Primer Pair Mix. The swab extract will be added at Step 6.

5. Vortex the PCR amplification mix for 5–10 seconds, then pipet 10.5µl of PCR amplification mix into each reaction well.

Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or locus-to-locus imbalance.

6. Pipet 2.0µl of swab extract for each sample into the appropriate well of the reaction plate.

7. For the positive amplification control, vortex the tube of 2800M Control DNA, then dilute an aliquot to 5.0ng/µl. Add 2µl (10ng) to a reaction well containing 10.5µl of PCR amplification mix.

   Note: Optimization of the amount of 2800M Control DNA may be required, depending on thermal cycling conditions and laboratory preferences.

4.C Direct Amplification of DNA from Swabs in a 12.5µl Reaction Volume (continued)

8. For the negative amplification control, pipet 2.0µl of Water, Amplification Grade, or TE^{-4} buffer instead of swab extract into a reaction well containing PCR amplification mix.

   Note: Additional negative controls can be included. Assemble a reaction containing the swab extract prepared from a blank swab, or assemble a reaction where the SwabSolution™ Reagent is processed as a blank without a swab.

9. Seal or cap the plate, or close the tubes. **Optional:** Briefly centrifuge the plate to bring contents to the bottom of the wells and remove any air bubbles.

Thermal Cycling

Amplification and detection instrumentation may vary. You will need to optimize protocols including the amount of template DNA, cycle number (24–27 cycles), injection time and loading volume for your laboratory instrumentation. Testing at Promega shows that 25 cycles works well for a variety of sample types. Cycle number will need to be optimized in each laboratory for each sample type.

1. Place the MicroAmp® plate or reaction tubes in the thermal cycler.
2. Select and run the recommended protocol, which is provided below and in Figure 4. The total cycling time is approximately 1 hour.

   Notes:

   1. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.
   2. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a silver-plated or gold-plated sample block. The ramp speed is set after the thermal cycling run is started. When the Select Method Options screen appears, select "Max" for the ramp speed and enter the reaction volume.

Thermal Cycling Protocol
96°C for 1 minute, then:
96°C for 5 seconds 60°C for 1 minute for 25 cycles, then:
60°C for 10 minutes
4°C soak

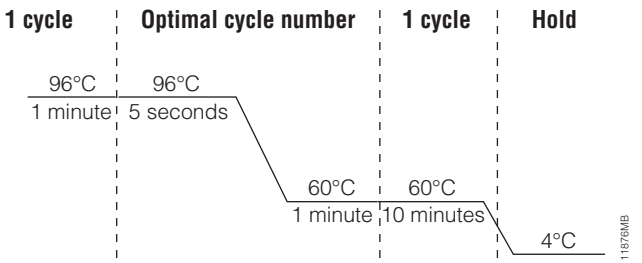

Figure 4. Thermal cycling protocol for the GeneAmp® PCR System 9700 and Veriti® 96-Well Thermal Cycler.

3. After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at –20°C in a light-protected box.

   Note: Long-term storage of amplified samples at 4°C or higher may produce artifacts.

PCR Optimization

Cycle number should be optimized based on the results of an initial experiment to determine the sensitivity with your collection method, sample types and instrumentation.

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.
2. Prepare four identical reaction plates with aliquots of the same swab extracts.
3. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (24–27 cycles).
4. Following amplification, use your laboratory's validated separation and detection protocols to determine the optimal cycle number for the sample type.

5. Instrument Setup and Sample Preparation

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer

Materials to Be Supplied by the User

- 95°C dry heating block, water bath or thermal cycler
- crushed ice, ice-water bath or a freezer plate block
- centrifuge compatible with 96-well plates
- aerosol-resistant pipette tips
- 3500/3500xL capillary array, 36cm
- plate retainer & base set (standard)
- POP-4® polymer for the 3500 or 3500xL
- anode buffer container
- cathode buffer container
- MicroAmp® optical 96-well plate and septa, or equivalent
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)

The quality of formamide is critical. Use Hi-Di™ formamide. Freeze formamide in aliquots at –20°C. Multiple freeze-thaw cycles or long-term storage at 4°C may cause breakdown of formamide. Poor-quality formamide may contain ions that compete with DNA during injection, which results in lower peak heights and reduced sensitivity. A longer injection time may not increase the signal.

Formamide is an irritant and a teratogen; avoid inhalation and contact with skin. Read the warning label, and take appropriate precautions when handling this substance. Always wear gloves and safety glasses when working with formamide.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer (continued)

Sample Preparation

1. At the first use, thaw the WEN Internal Lane Standard 500 and PowerPlex® Fusion 6C Allelic Ladder Mix completely. After the first use, store the reagents at 2–10°C.

   Note: Centrifuge tubes briefly to bring contents to the bottom, then vortex for 15 seconds before each use. Do not centrifuge after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Prepare a loading cocktail by combining and mixing WEN Internal Lane Standard 500 and Hi-Di™ formamide as follows:

   [(0.5µl WEN ILS 500) × (# samples)] + [(9.5µl Hi-Di™ formamide) × (# samples)]

   Note: The volume of internal lane standard used in the loading cocktail can be increased or decreased to adjust the intensity of the size standard peaks based on laboratory preferences. Adjust the volume added to the wells in Step 4 accordingly. Do not add less than 9.5µl of formamide per well.

3. Vortex for 10–15 seconds to mix.

4. Pipet 10µl of formamide/internal lane standard mix into each well.

5. Add 1µl of amplified sample (or 1µl of PowerPlex® Fusion 6C Allelic Ladder Mix) to each well. Cover wells with appropriate septa.

   Notes:

   1. Instrument detection limits vary; therefore, injection time or the amount of sample mixed with loading cocktail may need to be increased or decreased. To modify the injection time in the run module, select "Instrument Protocol" from the Library menu in the data collection software. If peak heights are higher than desired, use less DNA template in the amplification reactions or reduce the number of cycles in the amplification program to achieve the desired signal intensity. If the injection time is reduced, a decreased peak amplitude threshold for the orange channel may be required for proper sizing.
   2. Use a volume of allelic ladder that results in peak heights that are all consistently above the peak amplitude threshold determined as part of your internal validation.

6. Centrifuge plate briefly to remove air bubbles from the wells.

7. Denature samples at 95°C for 3 minutes, then immediately chill on crushed ice or a freezer plate block or in an ice-water bath for 3 minutes. Denature samples just prior to loading the instrument.

Instrument Preparation

Refer to the Applied Biosystems 3500/3500xL Genetic Analyzer User Guide for the instrument maintenance schedule and instructions to install the capillary array, buffers and polymer pouch and perform a spatial calibration. Samples may be analyzed as described in the *Applied Biosystems 3500/3500xL Genetic Analyzer User Guide*.

1. Open the 3500 Data Collection Software. The Dashboard screen will launch (Figure 5). To ensure that you are viewing the most up-to-date information, press the Refresh button. Ensure that the Consumables Information and Maintenance Notifications are acceptable.

   Set the oven temperature to 60°C, then select "Start Pre-Heat". When the Oven Temperature and Detection Cell Temperature turn green, you may proceed with the first injection.

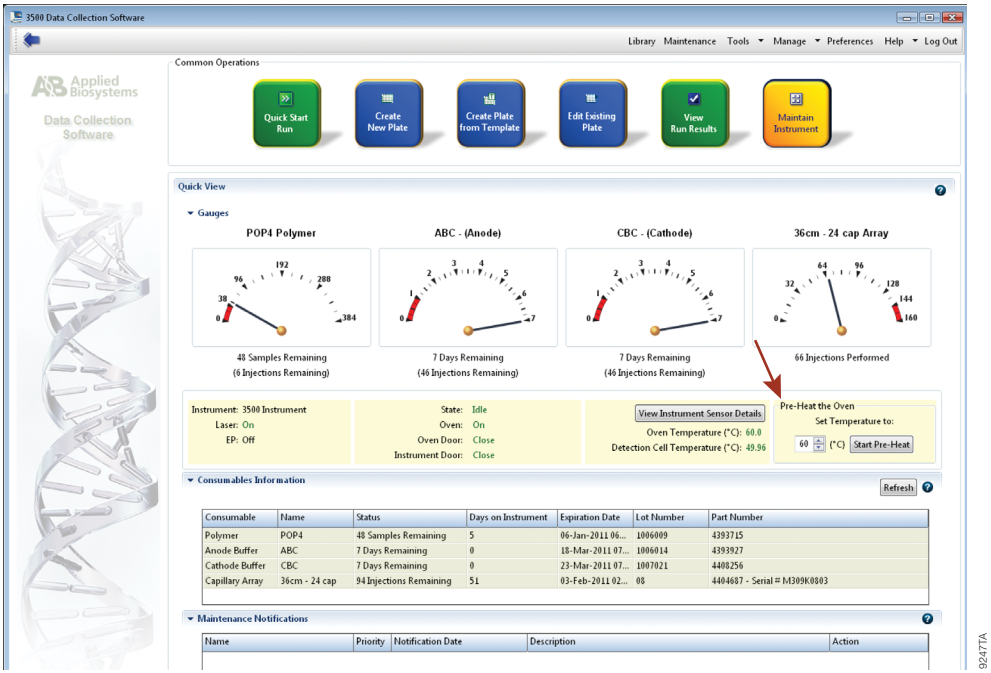

Figure 5. The Dashboard.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer (continued)

2. Prior to the first analysis using the PowerPlex® Fusion 6C System, an Instrument Protocol, Size Standard, QC Protocol, Assay, File Name Convention and Results Group must be created.

   a. To create a new Instrument Protocol, navigate to the Library, select "Instrument Protocols", then select "Create". Alternatively, a previously created Instrument Protocol may be used.

      Figure 6 shows the settings used at Promega for the Applied Biosystems® 3500xL Genetic Analyzer for the application type, dye set, capillary length, polymer, run module and appropriate protocol information.

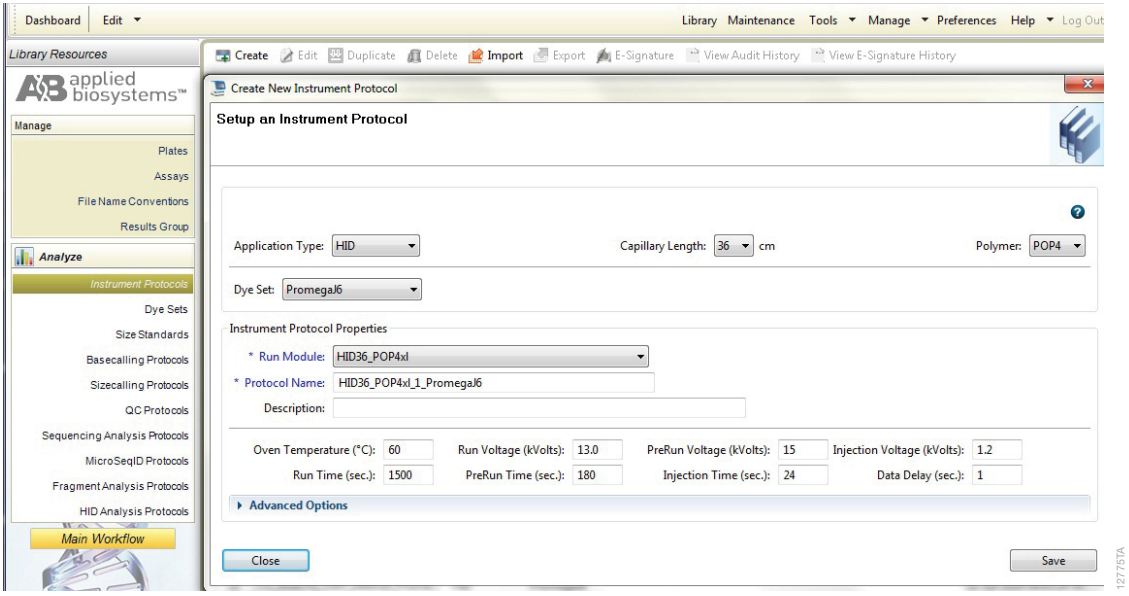

Figure 6. The Create New Instrument Protocol window.

The recommended settings are:

Application Type	HID
Capillary Length	36cm
Polymer	POP-4®
Dye Set	Promega J6
Run Module	HID36_POP4(xl)
Injection Time[1]	15 seconds for the Applied Biosystems® 3500 Genetic Analyzer 24 seconds for the Applied Biosystems® 3500xL Genetic Analyzer
Injection Voltage	1.2kV
Run Voltage	13kV
Run Time	1,500 seconds

[1]Injection time may be modified to increase or decrease peak heights.

When creating an Instrument Protocol, be sure to select the same dye set that was used to perform the Promega J6 spectral calibration.

Run time and other instrument settings should be optimized and validated in your laboratory.

When optimizing injection conditions in your laboratory, you may choose to create specific Instrument Protocols for each condition tested. If a single Instrument Protocol is used, follow the instructions in the *Applied Biosystems 3500/3500xL Genetic Analyzers User Guide* to edit a library entry.

Assign a descriptive protocol name.

Note: For more detailed information refer to the *Applied Biosystems 3500/3500xL Genetic Analyzers User Guide*.

b. To create a new Size Standard for the QC protocol, navigate to the Library. Select "Size Standards", then select "Create". Alternatively, a previously created Size Standard may be used.

Assign the Size Standard a descriptive name such as "WEN ILS 500". Choose "Orange" as the Dye Color. The fragments in the size standard are 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 and 500 bases. See Figure 7.

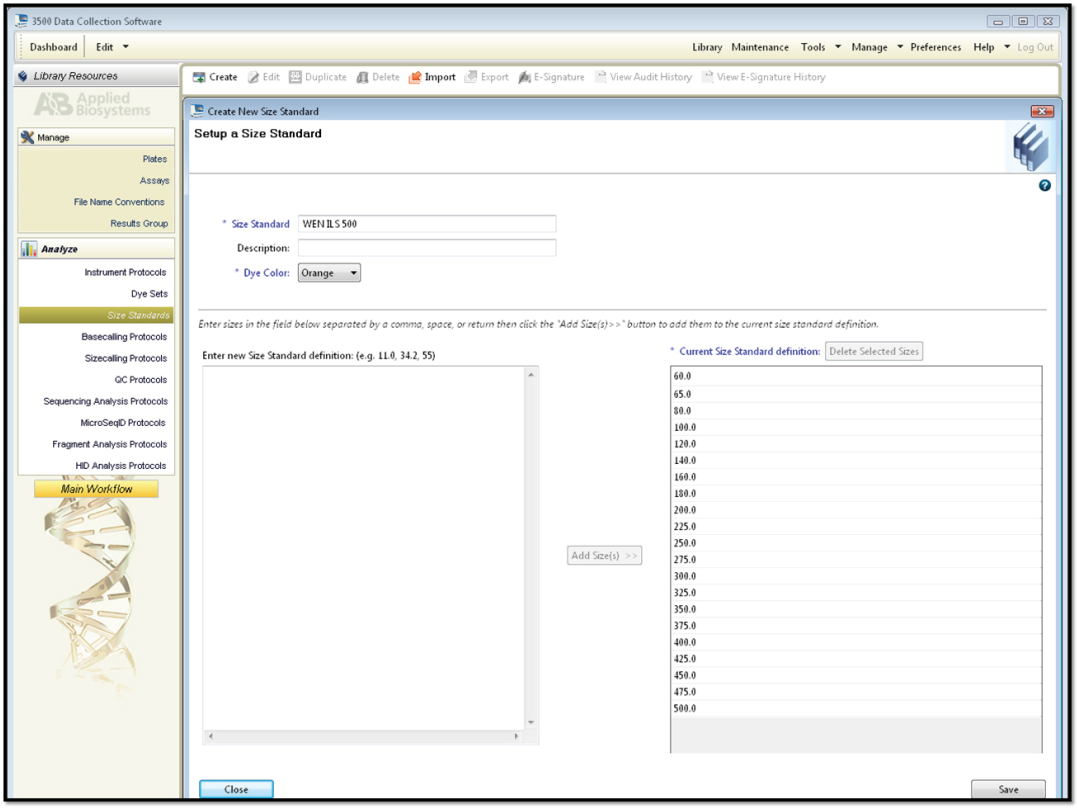

Figure 7. The Create New Size Standard window.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer (continued)

c. To create a new QC Protocol, navigate to the Library. Select "QC Protocols", then select "Create". Alternatively, a previously created QC Protocol may be used.

Assign a descriptive protocol name such as WEN ILS 500. Select the size standard created in Step 2.b. The settings for the QC protocol should be based on the internally validated conditions for the PowerPlex® Fusion 6C System on the Applied Biosystems® 3500 or 3500xL Genetic Analyzer. Figure 8 shows one option for these settings.

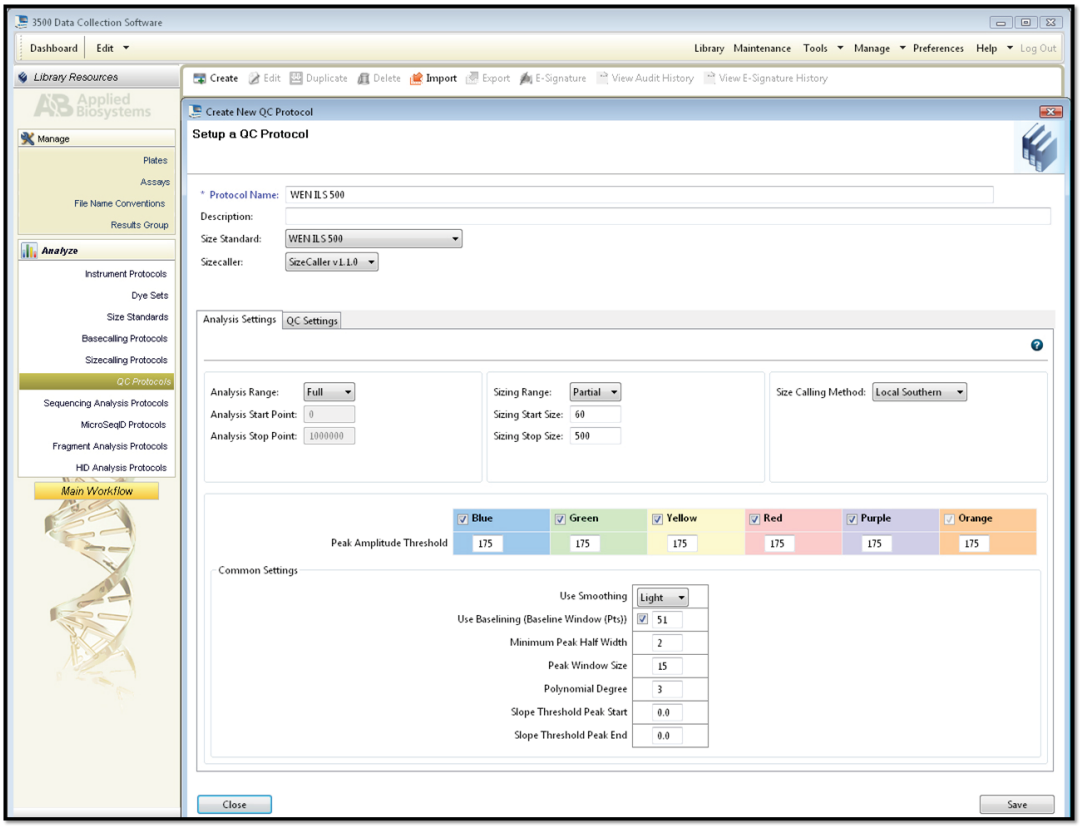

Figure 8. The Create New QC Protocol window.

d. To create a new Assay, navigate to the Library. Select “Assays”, then select “Create”. Alternatively, a previously created Assay may be used.

In the Create New Assay window (Figure 9), select the Instrument Protocol created in Step 2.a and the QC Protocol created in Step 2.c. Assign a descriptive assay name. Select the application type “HID”. An Assay is required for all named samples on a plate.

Note: If autoanalysis of sample data is desired, refer to the instrument user’s manual for instructions.

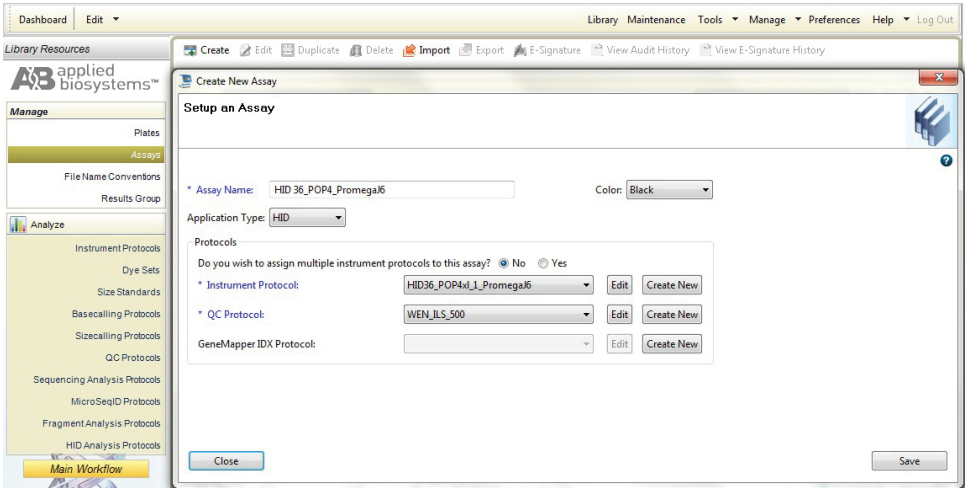

Figure 9. The Create New Assay window.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer (continued)

e. To create a new File Name Convention (Figure 10), navigate to the Library. Select "File Name Conventions", then select "Create". Alternatively, a previously created File Name Convention may be used.

Select the File Name Attributes according to your laboratory practices, and save with a descriptive name.

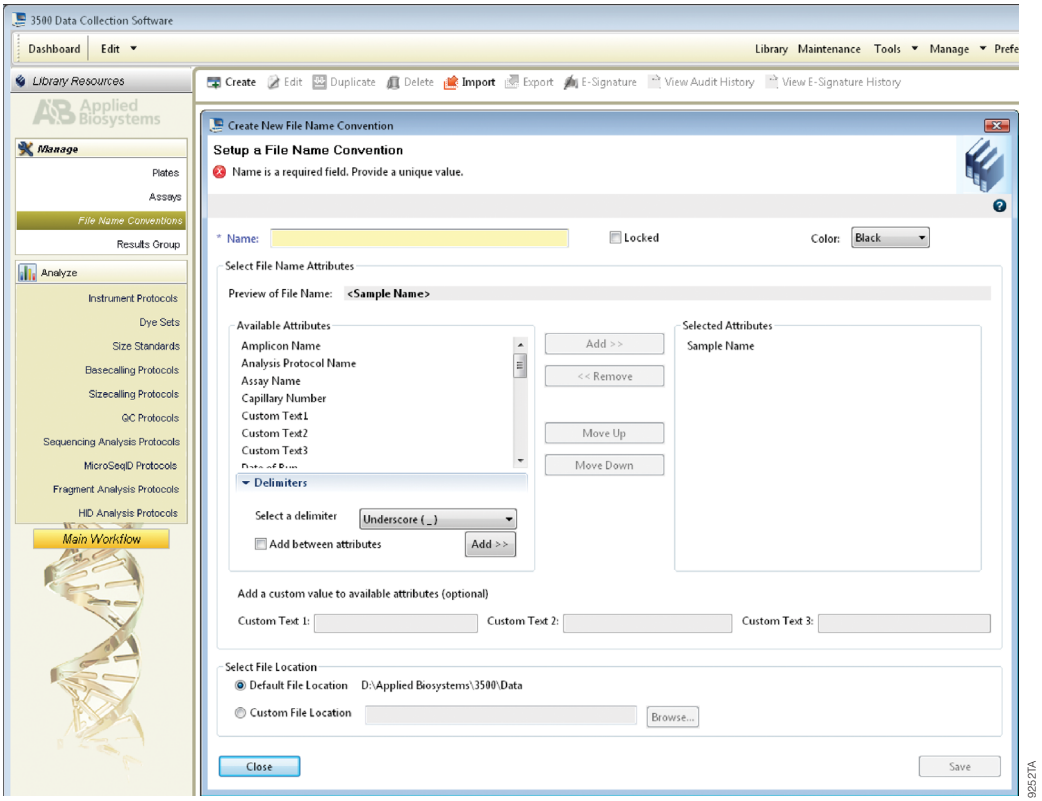

Figure 10. The Create New File Name Convention window.

f. To create a new Results Group (Figure 11), navigate to the Library. Select “Results Group”, then select “Create”. Alternatively, a previously created Results Group may be used.

Select the Results Group Attributes according to your laboratory practices. Save with a descriptive name.

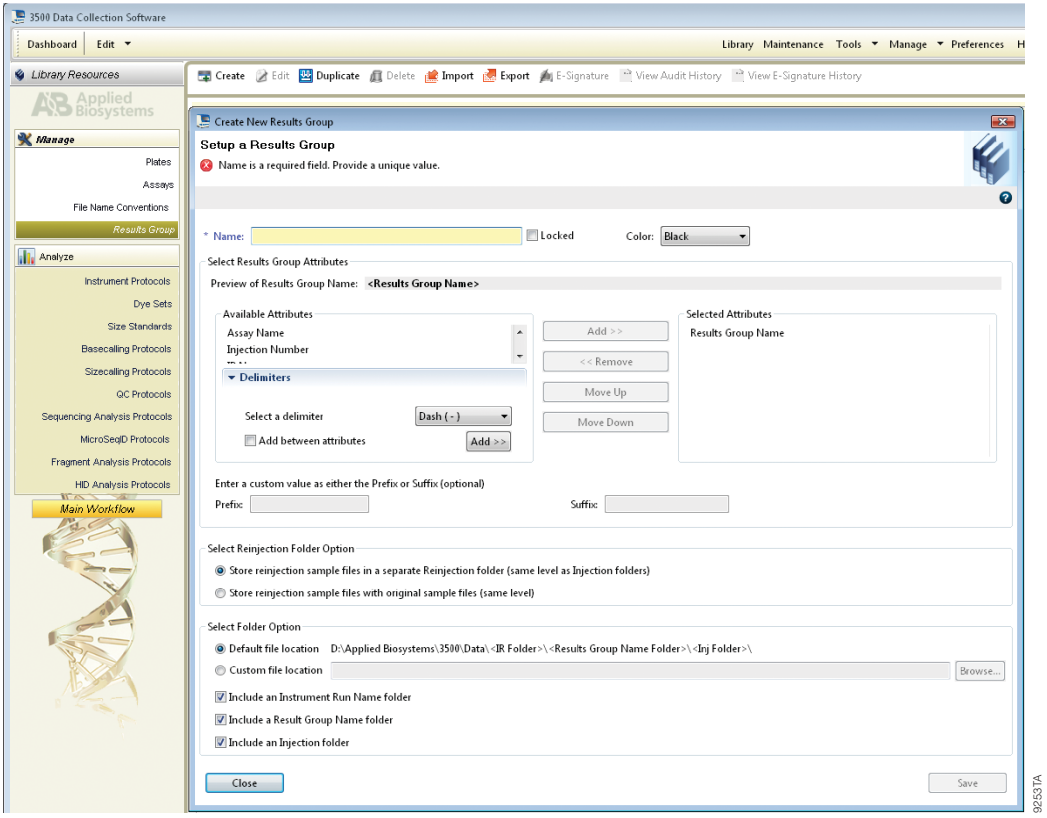

Figure 11. The Create New Results Group window.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer (continued)

3. To create a New Plate, navigate to the Library, and from the Manage menu, select "Plates", then "Create".

4. Assign a descriptive plate name. Select the plate type "HID" from the drop-down menu (Figure 12).

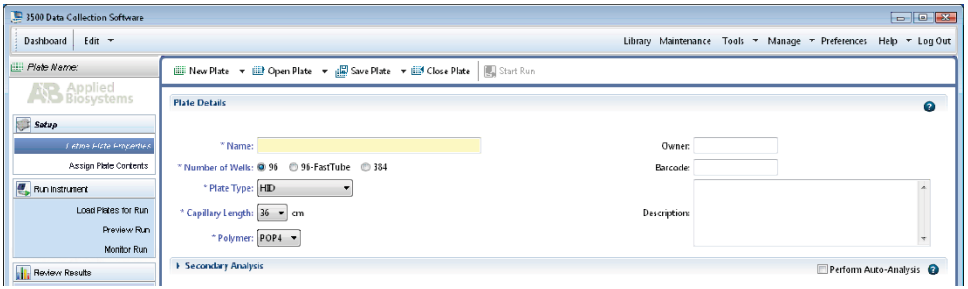

Figure 12. Defining plate properties.

5. Select "Assign Plate Contents" (Figure 13).

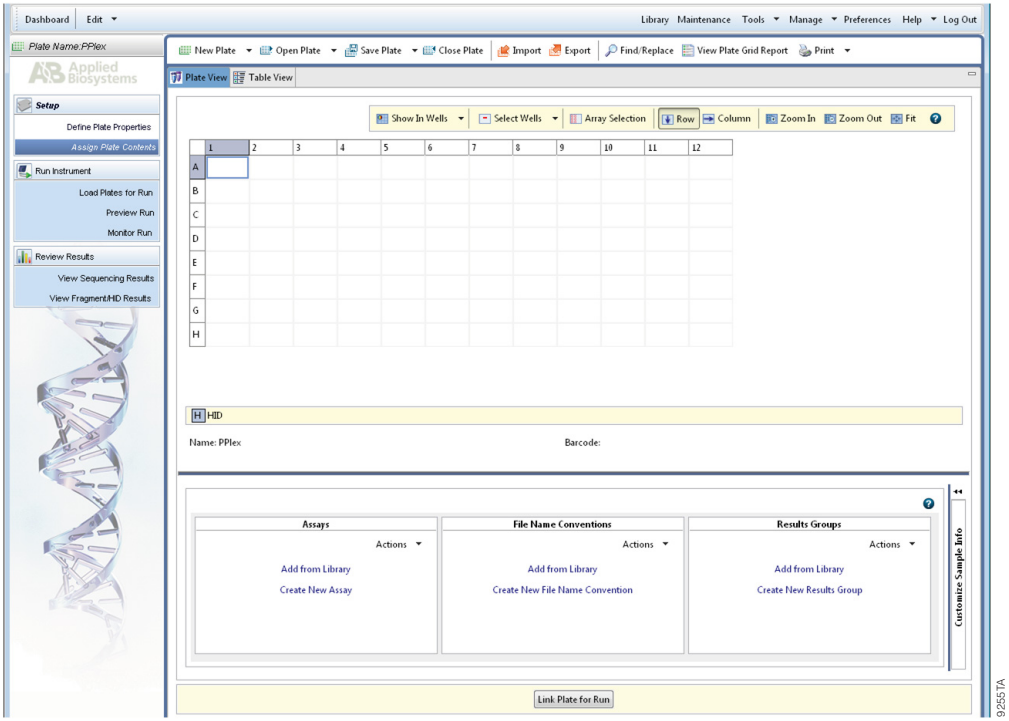

Figure 13. Assigning plate contents.

6. Assign sample names to wells.
7. In the lower left portion of the screen, under “Assays”, use the Add from Library option to select the Assay created in Step 2.d or one previously created. Click on the Add to Plate button, and close the window.
8. Under “File Name Conventions”, use the Add from Library option to select the File Name Convention created in Step 2.e or one previously created. Click on the Add to Plate button, and close the window.
9. Under “Results Groups”, use the Add from Library option to select the Results Group created in Step 2.f or one previously created. Click on the Add to Plate button, and close the window.
10. Highlight the sample wells, then select the boxes in the Assays, File Name Conventions and Results Groups that pertain to those samples.
11. Select “Link Plate for Run”.
12. The Load Plate window will appear. Select “Yes”.
13. In the Run Information window (Figure 14), assign a Run Name. Select “Start Run” (not shown).

    Each injection will take approximately 40 minutes.

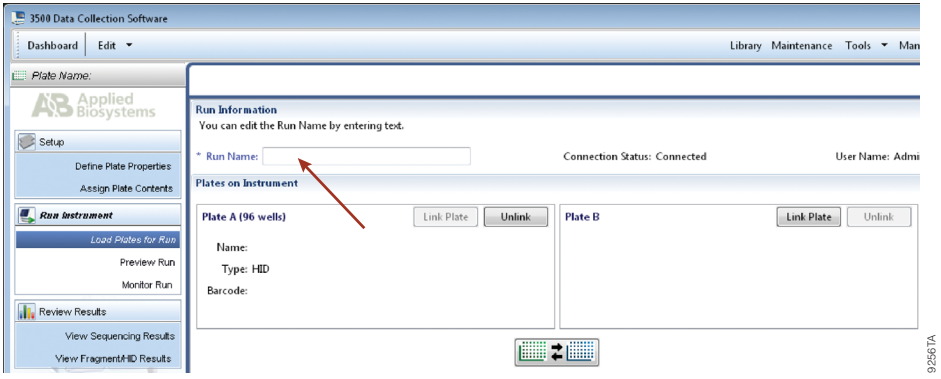

Figure 14. Assigning a run name.

5.B. Detection of Amplified Fragments Using the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with Data Collection Software Version 4.0 and DC v4 6-Dye Module v1 License

Materials to Be Supplied by the User

- 95°C dry heating block, water bath or thermal cycler
- crushed ice, ice-water bath or freezer plate block
- centrifuge compatible with 96-well plates
- aerosol-resistant pipette tips
- 3100 or 3130 capillary array, 36cm
- plate retainer & base set (standard)
- POP-4® polymer for the 3130/3130*xl* Genetic Analyzers
- 10X genetic analyzer buffer with EDTA
- MicroAmp® optical 96-well plate and septa, or equivalent
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)

The quality of formamide is critical. Use Hi-Di™ formamide. Freeze formamide in aliquots at –20°C. Multiple freeze-thaw cycles or long-term storage at 4°C may cause breakdown of formamide. Poor-quality formamide may contain ions that compete with DNA during injection, which results in lower peak heights and reduced sensitivity. A longer injection time may not increase the signal.

Formamide is an irritant and a teratogen; avoid inhalation and contact with skin. Read the warning label, and take appropriate precautions when handling this substance. Always wear gloves and safety glasses when working with formamide.

Sample Preparation

1. At the first use, thaw the WEN Internal Lane Standard 500 and PowerPlex® Fusion 6C Allelic Ladder Mix completely. After the first use, store the reagents at 2–10°C.

   Note: Centrifuge tubes briefly to bring contents to the bottom, then vortex for 15 seconds before each use. Do not centrifuge after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Prepare a loading cocktail by combining and mixing WEN Internal Lane Standard 500 and Hi-Di™ formamide as follows:

   [(0.5µl WEN ILS 500) × (# samples)] + [(9.5µl Hi-Di™ formamide) × (# samples)]

   Note: The volume of internal lane standard used in the loading cocktail can be increased or decreased to adjust the intensity of the size standard peaks based on laboratory preferences. Adjust the volume added to the wells in Step 4 accordingly. Do not use less than 9.5µl of formamide per well.

3. Vortex for 10–15 seconds to mix.

4. Pipet 10µl of formamide/internal lane standard mix into each well.

5. Add 1µl of amplified sample (or 1µl of PowerPlex® Fusion 6C Allelic Ladder Mix) to each well. Cover wells with appropriate septa.

   Note: Instrument detection limits vary; therefore, injection time, injection voltage or the amount of sample mixed with loading cocktail may need to be adjusted. Use the Module Manager in the data collection software to modify the injection time or voltage in the run module (see Instrument Preparation below). If the injection time or voltage is reduced, a decreased peak amplitude threshold for the orange channel may be required for proper sizing.

6. Centrifuge plate briefly to remove air bubbles from the wells.

7. Denature samples at 95°C for 3 minutes, then immediately chill on crushed ice or a freezer plate block or in an ice-water bath for 3 minutes. Denature samples just prior to loading the instrument.

Instrument Preparation

Refer to the instrument user's manual for instructions on cleaning, installing the capillary array, performing a spatial calibration and adding polymer.

Analyze samples as described in the user's manual for the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer, with the following exceptions.

1. In the Module Manager, select "New". Select "Regular" in the Type drop-down list, and select "HIDFragmentAnalysis36_POP4" in the Template drop-down list. Confirm that the injection time is 5 seconds, the injection voltage is 3kV and the run time is 1,500 seconds. Give a descriptive name to your run module, and select "OK".

   Note: Instrument sensitivities can vary. The injection time and voltage may be adjusted in the Module Manager. A suggested range for the injection time is 3–22 seconds and for the injection voltage is 1–3kV.

2. In the Protocol Manager, select "New". Type a name for your protocol. Select "Regular" in the Type drop-down list, and select the run module you created in the previous step in the Run Module drop-down list. Lastly, select "J6" in the dye-set drop-down list. Select "OK".

3. In the Plate Manager, create a new plate record as described in the instrument user's manual. In the dialog box that appears, select "GeneMapper—Generic" in the Application drop-down list, and select the appropriate plate type (96-well). Add entries in the owner and operator windows, and select "OK".

   Note: If autoanalysis of sample data is desired, refer to the instrument user's manual for instructions.

4. In the GeneMapper® plate record, enter sample names in the appropriate cells. Scroll to the right. In the Results Group 1 column, select the desired results group. In the Instrument Protocol 1 column, select the protocol you created in Step 2. Be sure this information is present for each row that contains a sample name. Select "OK".

   Note: To create a new results group, select "New" in the drop-down menu in the Results Group column. Select the General tab, and enter a name. Select the Analysis tab, and select "GeneMapper—Generic" in the Analysis type drop-down list.

5. Place samples in the instrument, and close the instrument doors.

5.B. Detection of Amplified Fragments Using the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with Data Collection Software Version 4 and DC v4 6-Dye Module v1 License (continued)

6. In the spectral viewer, select dye set J6, and confirm that the active dye set is the file generated for the PowerPlex® 6C-dye chemistry.

It is critical to select the correct J6 spectral for the PowerPlex® 6C-dye chemistry.

If the PowerPlex® 6C-dye chemistry is not the active dye set, locate the PowerPlex® 6C-dye spectral in the List of Calibrations for Dye Set J6, and select "Set".

7. In the run scheduler, locate the plate record that you just created in Steps 3 and 4, and click once on the name to highlight it.
8. Once the plate record is highlighted, click the plate graphic that corresponds to the plate on the autosampler that contains your amplified samples.
9. When the plate record is linked to the plate, the plate graphic changes from yellow to green, and the green Run Instrument arrow will become enabled.
10. Click on the green Run Instrument arrow on the toolbar to start the sample run.
11. Monitor electrophoresis by observing the run, view, array or capillaries viewer window in the data collection software. Each injection will take approximately 40 minutes.

6. Data Analysis Using GeneMapper® *ID*-X Software, Version 1.4

The instructions in this section were written using GeneMapper® software, version 1.4. Due to potential differences between individual software versions, some of the instructions may not apply to other software versions.

6.A. Importing PowerPlex® Fusion 6C Panels, Bins and Stutter Text Files into GeneMapper® *ID*-X Software, Version 1.4

To facilitate analysis of data generated with the PowerPlex® Fusion 6C System, we have created panels and bins text files to allow automatic assignment of genotypes using GeneMapper® *ID*-X software. We recommend that users receive training from Applied Biosystems on the GeneMapper® *ID*-X software to familiarize themselves with proper operation of the software.

Note: The panels, bins and stutter text files mentioned here are compatible with earlier versions of the GeneMapper® *ID*-X software.

Getting Started

1. To obtain the proper panels, bins and stutter text files and WEN_ILS_500_IDX.xml file for the PowerPlex® Fusion 6C System go to: **www.promega.com/resources/tools/genemapper-id-software-panels-and-bin-sets/**
2. Select the PowerPlex® System that you are using, and select "GeneMapper ID-X". Enter your contact information, and select "Submit".

3. Save the PowerPlex_Fusion_6C_Panels_IDX_vX.x.txt, PowerPlex_Fusion_6C_Bins_IDX_vX.x.txt and PowerPlex_Fusion_6C_Stutter_IDX_vX.x.txt files, where "X.x" refers to the most recent version of the panels, bins and stutter text files, to a known location on your computer.
4. Save the WEN_ILS_500_IDX.xml file to a known location on your computer.

Importing Panels, Bins and Stutter Text Files

1. Open the GeneMapper® *ID*-X software.
2. Select "Tools", then "Panel Manager".
3. Highlight the Panel Manager icon in the upper left navigation pane.
4. Select "File", then "Import Panels".
5. Navigate to the panels text file downloaded in the Getting Started Section. Select the file, then "Import".
6. In the navigation pane, highlight the PowerPlex Fusion 6C panels folder that you just imported in Step 5.
7. Select "File", then "Import Bin Set".
8. Navigate to the bins text file downloaded in the Getting Started section. Select the file, then "Import".
9. In the navigation pane, highlight the PowerPlex 6C Fusion panels folder that you just imported in Step 5.
10. Select "File", then "Import Marker Stutter". A warning box will appear asking if you want to overwrite current values. Select "Yes".
11. Navigate to the stutter text file imported in the Getting Started section. Select the file, then "Import".
12. In the Panel Manager, check the boxes to indicate DYS391, DYS576 and DYS570 are Y-markers. See Figure 15. This option is not available for older versions of the GeneMapper® *ID*-X software.
13. At the bottom of the Panel Manager window, select "OK". This will save the panels, bins and stutter text files and close the window.

	Marker Name	Dye Color	Min Size	Max Size	Control Alleles	Marker	Comments	Y Marker	Ladder Alleles
1	AMEL	Blue	80.0	89.0	X,Y	9	none	☐	X,Y
2	D3S1358	Blue	90.0	151.0	17,18	4	none	☐	9,10,11,12,13,14,15,16,17,18,19,20
3	D1S1656	Blue	152.0	209.5	12,13	4	none	☐	9,10,11,12,13,14,14.3,15,15.3,16,16.3,17,17.3,18,18.3,19,19.3,20.3
4	D2S441	Blue	211.0	252.0	10,14	4	none	☐	8,9,10,11,11.3,12,13,14,15,16,17
5	D10S1248	Blue	254.0	302.5	13,15	4	none	☐	8,9,10,11,12,13,14,15,16,17,18,19
6	D13S317	Blue	304.5	357.0	9,11	4	none	☐	5,6,7,8,9,10,11,12,13,14,15,16,17
7	Penta E	Blue	362.0	482.0	7,14	5	none	☐	5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25
8	D16S539	Green	74.0	129.4	9,13	4	none	☐	4,5,6,7,8,9,10,11,12,13,14,15,16
9	D18S51	Green	131.0	217.5	16,18	4	none	☐	7,8,9,10,10.2,11,12,13,13.2,14,15,16,17,18,19,20,21,22,23,24,25,26,27
10	D2S1338	Green	221.5	304.0	22,25	4	none	☐	10,12,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28
11	CSF1PO	Green	313.0	366.5	12	4	none	☐	5,6,7,8,9,10,11,12,13,14,15,16
12	Penta D	Green	373.5	470.0	12,13	5	none	☐	2.2,3.2,5,6,7,8,9,10,11,12,13,14,15,16,17
13	TH01	Yellow	65.0	118.0	6,9.3	4	none	☐	3,4,5,6,7,8,9,9.3,10,11,13.3
14	VWA	Yellow	121.0	192.0	16,19	4	none	☐	10,11,12,13,14,15,16,17,18,19,20,21,22,23,24
15	D21S11	Yellow	197.0	266.5	29,31.2	4	none	☐	24,24.2,25,25.2,26,27,28,28.2,29,29.2,30,30.2,31,31.2,32,32.2,33,33.2,34,34.2,35,35.2,36,37,38
16	D7S820	Yellow	268.0	315.5	8,11	4	none	☐	5,6,7,8,9,10,11,12,13,14,15,16
17	D5S818	Yellow	317.5	380.0	12	4	none	☐	6,7,8,9,10,11,12,13,14,15,16,17,18
18	TPOX	Yellow	390.0	448.0	11	4	none	☐	4,5,6,7,8,9,10,11,12,13,14,15,16
19	D8S1179	Red	66.0	129.8	14,15	4	none	☐	7,8,9,10,11,12,13,14,15,16,17,18,19
20	D12S391	Red	130.1	190.5	18,23	4	none	☐	14,15,16,17,17.3,18,18.3,19,20,21,22,23,24,25,26,27
21	D19S433	Red	192.0	255.0	13,14	4	none	☐	5.2,6.2,8,9,10,11,12,12.2,13,13.2,14,14.2,15,15.2,16,16.2,17,17.2,18,18.2
22	SE33	Red	270.0	429.0	15,16	4	none	☐	4.2,6.3,8,9,10,11,12,13,14,15,16,17,18,19,20,20.2,21,21.2,22,22.2,23.2,24.2,25.2,26.2,27.2,28.2,29.2,30.2,31.2,32.2,33.2,34.2,35,36,37,39
23	D22S1045	Red	430.0	478.0	16	3	none	☐	7,8,9,10,11,12,13,14,15,16,17,18,19,20
24	DYS391	Purple	79.5	131.0	10	4	none	☑	5,6,7,8,9,10,11,12,13,14,15,16
25	FGA	Purple	134.0	299.0	20,23	4	none	☐	14,15,16,17,18,18.2,19,19.2,20,20.2,21,21.2,22,22.2,23,23.2,24,24.2,25,25.2,26,27,28,29,30,31.2,32.2,33.2,42.2,43.2,44.2,45.2,46.2,48.2,50.2
26	DYS576	Purple	302.0	370.0	18	4	none	☑	11,12,13,14,15,16,17,18,19,20,21,22,23
27	DYS570	Purple	380.0	464.0	17	4	none	☑	10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25

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Figure 15. The GeneMapper® *ID*-X Software, Version 1.4, Y-Marker Check Box.

6.B. Importing the WEN ILS 500 IDX Size Standard into GeneMapper® *ID*-X Software, Version 1.4

There are two options when creating a size standard. Use this protocol or the alternative protocol in Section 6.C.

1. Select "Tools", then "GeneMapper ID-X Manager".
2. Select the Size Standard tab.
3. Select "Import".
4. Navigate to the location of the WEN_ILS_500_IDX.xml file on your computer.
5. Highlight the file, then select "Import".
6. Select "Done" to save changes and close the GeneMapper® *ID*-X Manager.

6.C. Creating a Size Standard with GeneMapper® *ID*-X Software, Version 1.4

1. Select "Tools", then "GeneMapper ID-X Manager".
2. Select the Size Standard tab.
3. Select "New".
4. In the Size Standard Editor window (Figure 16), select "GeneMapper ID-X Security Group" as the Security Group. This allows access for all users of the software. Other security groups may be used.
5. Enter a detailed name, such as "WEN_ILS_500_IDX".
6. Choose "Orange" for the Size Standard Dye.
7. Enter the sizes of the internal lane standard fragments (60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 and 500 bases). See Section 11.C, Figure 29.
8. Select "OK".

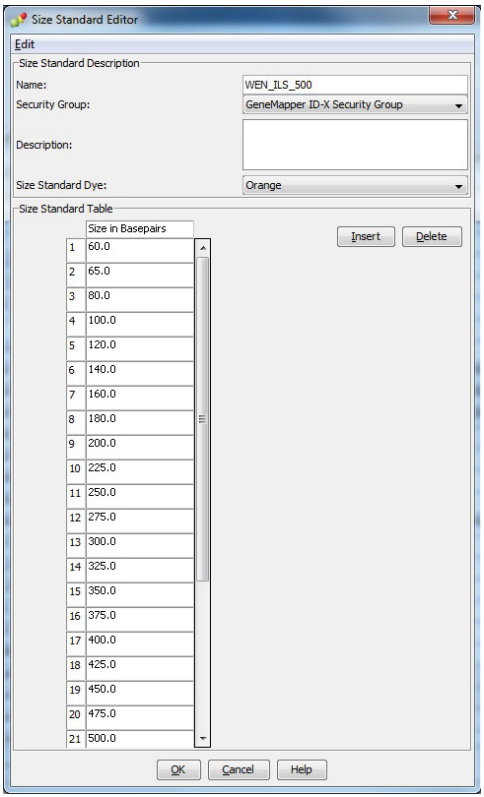

Figure 16. The GeneMapper® *ID*-X Software, Version 1.4, Size Standard Editor.

6.D. Creating a Casework Analysis Method with GeneMapper® *ID*-X Software, Version 1.4

These instructions are intended as a guide to start analyzing data in GeneMapper® *ID*-X software. They are not intended as a comprehensive guide for using GeneMapper® *ID*-X software. We recommend that users contact Applied Biosystems for training on the software.

1. Select "Tools", then "GeneMapper ID-X Manager".
2. Select the Analysis Methods tab.
3. Select "New", and a new analysis method dialog box will open.
4. In the Analysis Method Editor window, select "GeneMapper ID-X Security Group" as the Security Group. This allows access for all users of the software. Other security groups may be used.
5. Enter a descriptive name for the analysis method, such as "PowerPlex Fusion 6C".
6. Select the Allele tab (Figure 17).

6.D. Creating a Casework Analysis Method with GeneMapper® *ID*-X Software, Version 1.4 (continued)

7. Select the bins text file that was imported in Section 6.A.

8. Ensure that the "Use marker-specific stutter ratio and distance if available" box is checked. Doing this will assign locus-specific stutter filters and distances from the imported stutter file. We recommend the settings shown in Figure 17 for proper filtering of stutter peaks when using the PowerPlex® Fusion 6C System.

   Note: If you do not check the "Use marker-specific stutter ratio and distance if available" box, you will need to optimize these settings. In-house validation should be performed.

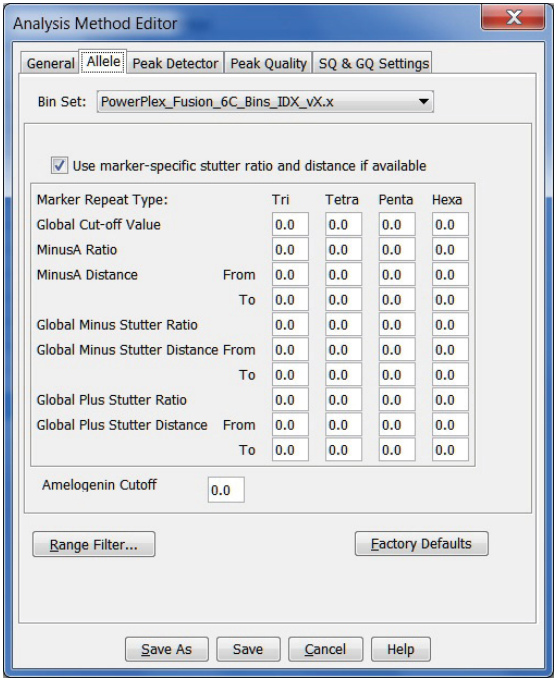

Figure 17. The GeneMapper® *ID*-X Software, Version 1.4, Allele tab.

9. Select the Peak Detector tab (Figure 18). You may need to optimize these settings. In-house validation should be performed.

   Notes:

   1. Select full range or partial range for the analysis range. When using a partial range, choose an appropriate analysis range based on your data. Choose a start point after the primer peak and just before the first defined internal lane standard peak to help ensure proper sizing of the internal lane standard.
   2. The peak amplitude thresholds are the minimum peak heights at which the software will call a peak. Values for peak amplitude thresholds are usually 50–150RFU for data generated on the Applied Biosystems® 3130 and 3130*xl* Genetic Analyzers. For the Applied Biosystems® 3500 and 3500xL Genetic Analyzers, Life Technologies suggests an analysis threshold of 175RFU under their default injection conditions. However, individual laboratories should determine their peak amplitude thresholds from internal validation studies.
   3. The normalization box can be checked regardless of whether normalization was or was not applied during data collection.

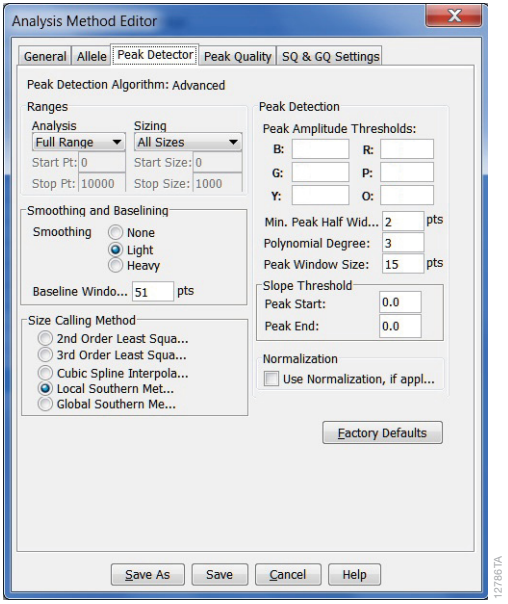

Figure 18. The GeneMapper® *ID*-X Software, Version 1.4, Peak Detector tab.

10. Select the Peak Quality tab. You may change the settings for peak quality.

    Note: For Steps 11 and 12, see the GeneMapper® *ID*-X user's manual for more information.

11. Select the SQ & GQ Settings tab. You may change these settings.
12. Select "Save" to save the new analysis method.
13. Select "Done" to exit the GeneMapper® *ID*-X Manager.

6.D. Creating a Casework Analysis Method with GeneMapper® *ID*-X Software, Version 1.4 (continued)

Processing Data for Casework Samples

1. Select "File", then "New Project".
2. Select "Edit", then "Add Samples to Project".
3. Browse to the location of the run files. Highlight desired files, then select "Add to list" followed by "Add".
4. In the Sample Type column, use the drop-down menu to select "Allelic Ladder", "Sample", "Positive Control" or "Negative Control" as appropriate for the sample. Every folder in the project must contain at least one allelic ladder injection that is designated as "Allelic Ladder" in the Sample Type column for proper genotyping.
5. In the Analysis Method column, select the analysis method created previously in this section.
6. In the Panel column, select the panels text file that was imported in Section 6.A.
7. In the Size Standard column, select the size standard that was imported in Section 6.B or created in Section 6.C.
8. Select "Analyze" (green arrow button) to start data analysis.

   Note: By default, the software is set to display the Analysis Requirements Summary window and Allelic Ladder Analysis Summary window if an issue is detected. After analysis is complete, the default setting is to show the Analysis Summary tab. If these default settings are changed, manual troubleshooting may be necessary.
9. If all analysis requirements are met, the Save Project window will open (Figure 19).

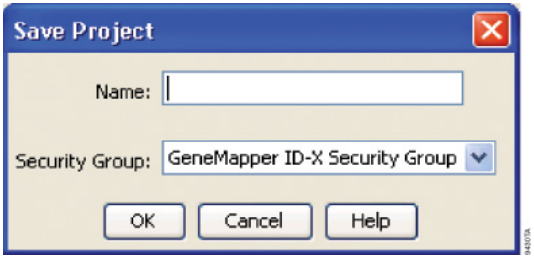

Figure 19. The Save Project window.

10. Enter the project name.
11. Choose the applicable security group from the drop-down menu, then select "OK".

When the analysis is finished, the Analysis Summary screen will appear. We recommend that you review any yellow or red marker header bars in the plots view and handle them according to laboratory standard operating procedures.

The values displayed in the Analysis Method Peak Quality and SQ & GQ Settings tabs are defaults and will affect the quality values displayed in the plot settings. We recommend that you modify the values in these tabs to fit your laboratory's data analysis protocols.

6.E. Creating a Databasing or Paternity Analysis Method with GeneMapper® *ID*-X Software, Version 1.4

These instructions are intended as a guide to start analyzing data in GeneMapper® *ID*-X software. They are not intended as a comprehensive guide for using the GeneMapper® *ID*-X software. We recommend that users contact Applied Biosystems for training on the software.

1. Select "Tools", then "GeneMapper ID-X Manager".
2. Select the Analysis Methods tab.
3. Select "New", and a new analysis method dialog box will open.
4. In the Analysis Method Editor window, select "GeneMapper ID-X Security Group" as the Security Group. This allows access for all users of the software. Other security groups may be used.
5. Enter a descriptive name for the analysis method, such as "PowerPlex Fusion 6C 20% Filter".
6. Select the Allele tab (Figure 20).
7. Select the bins text file that was imported in Section 6.A.
8. Ensure that the "Use marker-specific stutter ratio and distance if available" box is checked. Doing this will assign locus-specific stutter filters and distances from the imported stutter file. Ensure that the appropriate global filter is applied to this analysis method. For example, for a 20% filter enter "0.20" for the Global Cut-off Value for Tri, Tetra and Penta repeats (Figure 20).

   Note: If you do not check the "Use marker-specific stutter ratio and distance if available" box, you will need to optimize these settings. In-house validation should be performed.

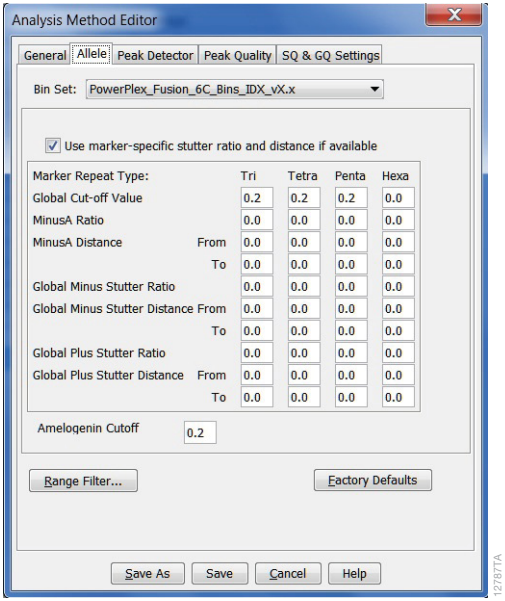

Figure 20. The GeneMapper® *ID*-X Software, Version 1.4, Allele tab with settings for using a 20% peak filter.

6.E. Creating a Databasing or Paternity Analysis Method with GeneMapper® *ID*-X Software, Version 1.4 (continued)

9. Select the Peak Detector tab (Figure 18). You will need to optimize these settings. In-house validation should be performed.

   Notes:

   1. Select full range or partial range for the analysis range. When using a partial range, choose an appropriate analysis range based on your data. Choose a start point after the primer peak and just before the first defined internal lane standard peak to help ensure proper sizing of the internal lane standard.
   2. The peak amplitude thresholds are the minimum peak heights at which the software will call a peak. Values for peak amplitude thresholds are usually 50–150RFU on the Applied Biosystems® 3130 and 3130*xl* Genetic Analyzers. For the Applied Biosystems® 3500 and 3500xL Genetic Analyzers, Life Technologies suggests an analysis threshold of 175RFU under their default injection conditions. However, individual laboratories should determine their peak amplitude thresholds from internal validation studies.
   3. The normalization box can be checked regardless of whether normalization was or was not applied during data collection.

10. Select the Peak Quality tab. You may change the settings for peak quality.

    Note: For Steps 10 and 11, see the GeneMapper® *ID*-X user's manual for more information.

11. Select the SQ & GQ Settings tab. You may change these settings.

12. Select "Save" to save the new analysis method.

13. Select "Done" to exit the GeneMapper® *ID*-X Manager.

Processing Data for Databasing or Paternity Samples

1. Select "File", then "New Project".

2. Select "Edit", then "Add Samples to Project".

3. Browse to the location of run files. Highlight desired files, then select "Add to list" followed by "Add".

4. In the Sample Type column, use the drop-down menu to select "Allelic Ladder", "Sample", "Positive Control" or "Negative Control" as appropriate for the sample. Every folder in the project must contain at least one allelic ladder injection that is designated as "Allelic Ladder" in the Sample Type column for proper genotyping.

5. In the Analysis Method column, select the analysis method created previously in this section.

6. In the Panel column, select the panels text file that was imported in Section 6.A.

7. In the Size Standard column, select the size standard that was imported in Section 6.B or created in Section 6.C.

8. Select "Analyze" (green arrow button) to start data analysis.

   Note: By default, the software is set to display the Analysis Requirements Summary window and Allelic Ladder Analysis Summary window if an issue is detected. After analysis is complete, the default setting is to show the Analysis Summary tab. If these default settings are changed, manual troubleshooting may be necessary.

9. If all analysis requirements are met, the Save Project window will open (Figure 19).

10. Enter the project name.

11. Choose the applicable security group from the drop-down menu, then select "OK".

When the analysis is finished, the Analysis Summary screen will appear. We recommend that you review any yellow or red marker header bars in the plots view and handle them according to laboratory standard operating procedures.

The values displayed in the Analysis Method Peak Quality and SQ & GQ Settings tabs are defaults and will affect the quality values displayed in the plot settings. We recommend that you modify the values in these tabs to fit your laboratory's data analysis protocols.

6.F. Controls in GeneMapper® *ID*-X Software

1. Observe the results for the negative control. Using the protocols defined in this manual, the negative control should be devoid of amplification products.

2. Observe the results for the 2800M Control DNA. The expected 2800M DNA allele designations for each locus are listed in Table 9 (Section 11.A).

7. Data Analysis Using GeneMarker® HID Software, Version 2.7.1

The instructions in this section were written using GeneMarker® HID software, version 2.7.1. Due to potential differences between individual software versions, some of the instructions may not apply to other software versions.

7.A. Creating an Analysis Method with GeneMarker® HID Software, Version 2.7.1

These instructions are intended as a guide to start analyzing data in GeneMarker® HID Software. They are not intended as a comprehensive guide for using GeneMarker® HID Software. We recommend that users contact SoftGenetics at: **www.softgenetics.com** for training and technical support for the software.

Contact SoftGenetics to obtain the current PowerPlex® Fusion 6C stutter, panels and bins files.

1. Open the GeneMarker® HID software.

2. To access your data files, select "Open Data" in the Magic Wizard (Figure 21), and click Add.

Figure 21. The GeneMarker® HID Magic Wizard.

3. Navigate to the directory containing your raw data files, and select the desired files.

4. Select "Open", and the selected files will appear in the Data File List.

   Note: Be sure that the Auto-Elevate box is not checked.

7.A. Creating an Analysis Method with GeneMarker® HID Software, Version 2.7.1 (continued)

5. The first time that you use the PowerPlex® Fusion 6C System with the GeneMarker® HID software you will need to set the dye channels. To do so, click the Channels button in the bottom left of the Data Files window, then select the 6 Colors tab. Set the dye channels as shown in Figure 22.

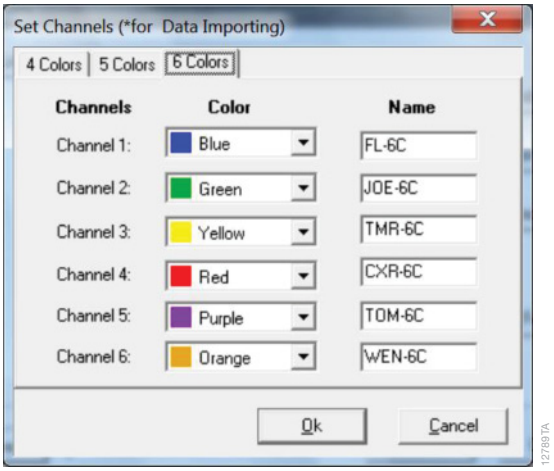

Figure 22. The GeneMarker® HID dye channels.

6. Select "OK" in the Open Data Files window, and the data will be uploaded into the GeneMarker® HID Software. In the Raw Data Tree, verify that the sample types (allelic ladder, positive control and negative control) are designated.

   If sample types are not designated, designate sample types by right clicking on the file name and selecting "Set sample type".

   Note: Sample types can be designated in the file name. See Step 8.b.

7. Use the Panel Editor in the Tools menu to select a PowerPlex® Fusion 6C panel. Click on the plus symbol to expand the list, then right click on a marker, and select "Edit". Enter laboratory-specified values for Min Homozygote Intensity, Min Heterozygote Intensity and Min Heterozygote Imbalance (Figure 23). This will set values for peaks within the marker range.

   Values for peak amplitude thresholds are usually 50–150RFU for data generated on the Applied Biosystems® 3130 and 3130*xl* Genetic Analyzers. For the Applied Biosystems® 3500 and 3500xL Genetic Analyzers, Life Technologies suggests an analysis threshold of 175RFU under their default injection conditions. However, individual laboratories should determine their peak amplitude thresholds from internal validation studies.

   These settings can be applied to all markers by checking the Apply Homo/Hetero Settings to All Markers checkbox.

   Select "OK", close the Panel Editor window and select "Save Changes".

   Note: Panels with different analysis values can be created by selecting "Save as New Panel" from the File menu.

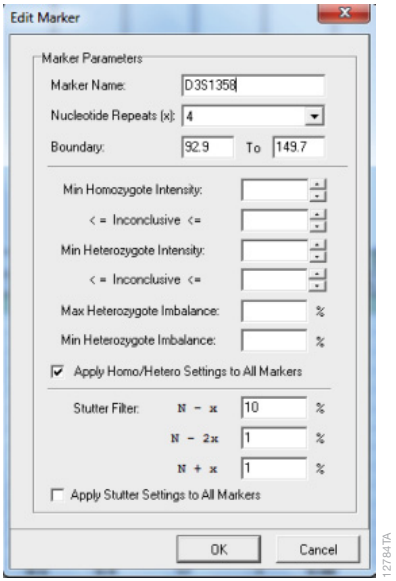

Figure 23. The GeneMarker® HID Edit Marker window for the D3S1358 marker.

8. In the View menu, select "Preferences…".

   a. Navigate to the Forensic tab. Select "Auto-Delete Alleles in Variant Bins in Allelic Ladder", and enter sample name identifiers for ladder, positive and negative controls.

   b. Navigate to the Display Settings tab, Sample Tree section, check boxes for "Flag Low-Quality ILS as 'SQ'" and "Consider Gender for Flag '?'". In the Allele Label section, uncheck "Flag Variant Alleles in Ladder".

   c. Select "OK".

7.A. Creating an Analysis Method with GeneMarker® HID Software, Version 2.7.1 (continued)

9. Click the Run Project icon (green arrow) in the toolbar, and the Template Selection window will appear. Select the PowerPlex_6C_Fusion template and the settings shown in Figure 24. Choose the panel that you created in Step 7 from the drop-down menu. Verify selection of Size Standard ILS_500 and Standard Color WEN. Select "Next".

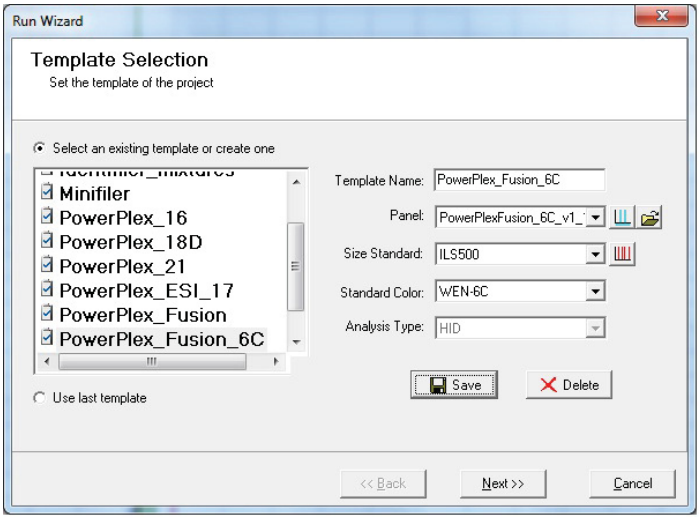

Figure 24. The Template Selection window.

10. The Data Process window will appear (Figure 25). Choose settings based on your laboratory's standard operating procedures. Select full range or partial range for the analysis range. When using a partial range, choose an appropriate analysis range based on your data. For the allelic call range choose a start point before the first defined internal lane standard peak and an end point just beyond the last defined internal lane standard peak.

    The peak detection threshold is the minimum peak height at which the software will call a peak **outside the marker range**.

    Select "Next".

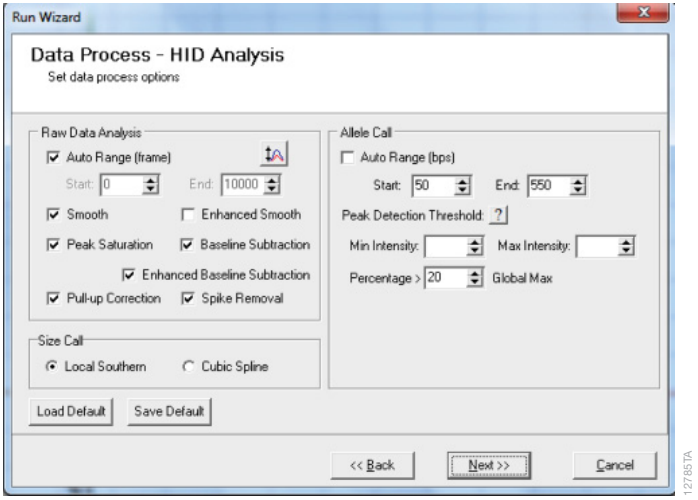

Figure 25. The Data Process window for an analysis method.

7.A. Creating an Analysis Method with GeneMarker® HID Software, Version 2.7.1 (continued)

11. The Additional Settings window will appear. Select the settings shown in Figure 26. The values displayed in the Allele Evaluation dialogue box are defaults and will affect the quality values displayed in the plot settings. We recommend that you modify the values in these tabs to fit your laboratory's data analysis protocols. Select "OK".

    Note: The Auto Select Best Ladder function will analyze each sample file with the passing ladder that most closely matches. If this box in not checked, select an appropriate ladder in the Allelic Ladder drop-down menu.

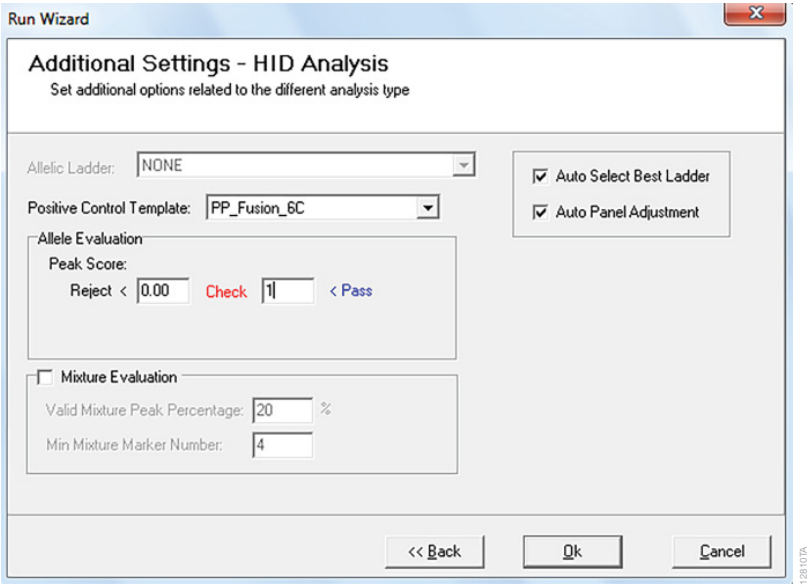

Figure 26. The Additional Settings window.

12. When the analysis is finished, the Main Analysis window will appear. We recommend that you review any yellow or red flagged markers in the Report Table window and handle them according to your laboratory's standard operating procedures.

7.B. Controls in the GeneMarker® HID Software

1. Observe the results for the negative control. Using the protocols defined in the manual, the negative controls should be devoid of amplification products.

2. Observe the results for the 2800M Control DNA. The expected 2800M allele designations for each locus are listed in Table 9 (Section 11.A). A file with the correct PowerPlex® Fusion 6C profile for the 2800M Control DNA should be preloaded in the GeneMarker® HID software.

   If the 2800M profile is not preloaded in the GeneMarker® HID software, you can create a Positive Control Template. To do so, open "Tools" in the Main Analysis window, then select "Positive Control Template Editor". In the Positive Control Standards dialogue box select "Add". Enter a descriptive name for the control, such as "2800M_Fusion_6C", and select OK. In the Import Genotypes from Sample dialogue box, select the positive control sample. In the Confirm window, select "Yes". In the Positive Control Template Editor, select "OK".

8. Results

Representative results of the PowerPlex® Fusion 6C System are shown in Figure 27. The PowerPlex® Fusion 6C Allelic Ladder Mix is shown in Figure 28.

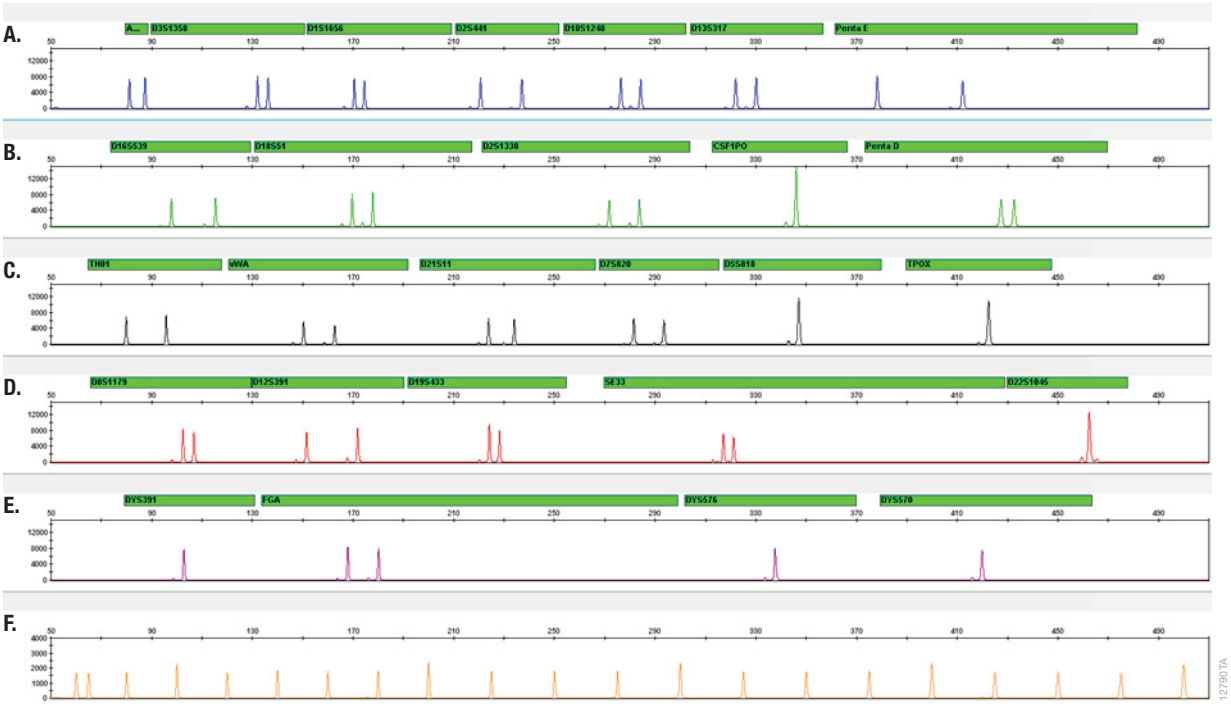

Figure 27. The PowerPlex® Fusion 6C System. The 2800M Control DNA (1.0ng) was amplified using the PowerPlex® Fusion 6C System and 29 cycles. Amplification products were mixed with WEN Internal Lane Standard 500 and analyzed using an Applied Biosystems® 3500 Genetic Analyzer and a 1.2kV, 15-second injection. Results were analyzed using GeneMapper® *ID*-X software, version 1.4. **Panel A.** An electropherogram showing the peaks of the FL-6C-labeled loci: Amelogenin, D3S1358, D1S1656, D2S441, D10S1248, D13S317 and Penta E. **Panel B.** An electropherogram showing the peaks of the JOE-6C-labeled loci: D16S539, D18S51, D2S1338, CSF1PO and Penta D. **Panel C.** An electropherogram showing the peaks of the TMR-6C-labeled loci: TH01, vWA, D21S11, D7S820, D5S818, and TPOX. **Panel D.** An electropherogram showing the peaks of the CXR-6C-labeled loci: D8S1179, D12S391, D19S433, SE33 and D22S1045. **Panel E.** An electropherogram showing the TOM-6C-labeled loci: DYS391, FGA, DYS576 and DYS570. **Panel F.** An electropherogram showing the 60bp to 500bp fragments of the WEN Internal Lane Standard 500.

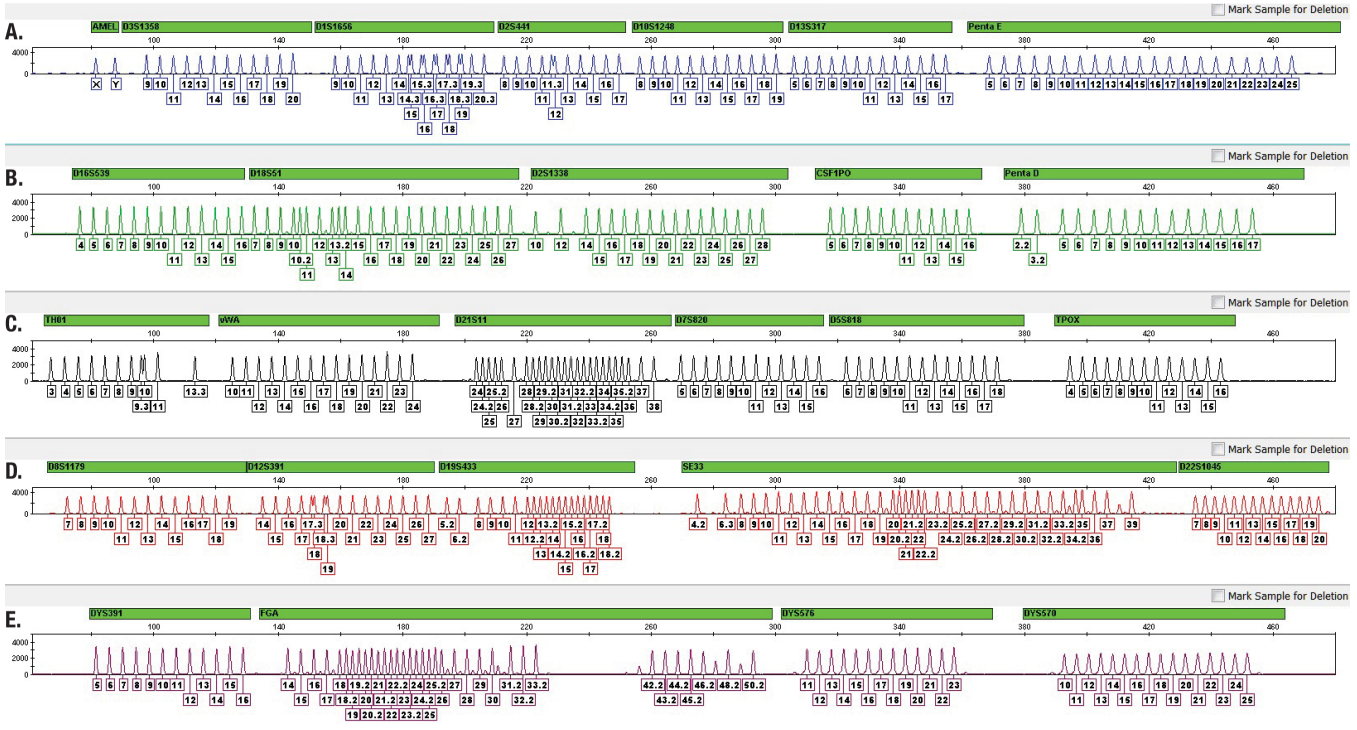

Figure 28. The PowerPlex® Fusion 6C Allelic Ladder Mix. The PowerPlex® Fusion 6C Allelic Ladder Mix was analyzed using an Applied Biosystems® 3500xL Genetic Analyzer and a 1.2kV, 24-second injection. The sample file was analyzed with the GeneMapper® *ID*-X software, version 1.4, and PowerPlex® Fusion 6C panels and bins text files. **Panel A.** The FL-6C-labeled allelic ladder components and their allele designations. **Panel B.** The JOE-6C-labeled allelic ladder components and their allele designations. **Panel C.** The TMR-6C-labeled allelic ladder components and their allele designations. **Panel D.** The CXR-6C-labeled allelic ladder components and their allele designations. **Panel E.** The TOM-6C-labeled allelic ladder components and their allele designations.

Artifacts and Stutter

Stutter products are a common amplification artifact associated with STR analysis. Stutter products often are observed one repeat unit smaller than the true allele peak and, occasionally, two repeat units smaller or one repeat unit larger than the true allele peak. Frequently, alleles with a greater number of repeat units will exhibit a higher percentage of stutter. A trinucleotide repeat locus, like D22S1045, will have more pronounced stutter in both n−3 and n+3 positions than a typical tetranucleotide repeat locus. The pattern and intensity of stutter may differ slightly between primer sets for the same loci.

The mean plus three standard deviations at each locus is used for locus-specific stutter filtering for the PowerPlex® Fusion 6C System.

In addition to stutter peaks, DNA-dependent artifact peaks (Table 4) and DNA-independent artifact peaks (Table 5) can be observed at some PowerPlex® Fusion 6C System loci.

Table 4. DNA-Dependent Artifacts Observed with the PowerPlex® Fusion 6C System.

Locus	Artifact Size
Amelogenin	n−1
D1S1656	n−1, n−2
D13S317	n−2
D18S51	n−2
vWA	n−2, elevated baseline in the locus
D7S820	n−2
D5S818	n−2
D19S433	n−2
SE33	n−2
DYS391	n−1
FGA	n−1, n−2

Table 5. DNA-Independent Artifacts Observed with the PowerPlex® Fusion 6C System.

Dye Label	Artifact Size[1]
FL-6C	~65–75bp
	~113–120bp
	~137–145bp
JOE-6C	~60–66bp
TMR-6C	~57–62bp

[1]Artifact sizes may vary depending on CE instrumentation and environmental conditions in the laboratory.

8. Results (continued)

Testing was performed with a variety of nonhuman DNA templates from bacteria, yeast, mammals and primates to characterize known artifacts with the PowerPlex® Fusion 6C System. The artifacts listed in Table 6 were noted above the 175RFU threshold with 10ng of template DNA using an Applied Biosystems® 3500xL Genetic Analyzer. Partial profiles were obtained with all primate species tested, but these profiles can be distinguished from a human profile because most of the alleles were called as off-ladder or were outside the locus panels.

Table 6. Nonhuman DNA Cross-Reactivity.

DNA Source	Artifact Size	Dye Label
Bovine	~98bp	FL-6C
Chicken	~221bp	JOE-6C
	~300bp	TMR-6C
Mouse	~347bp	JOE-6C
Pig	~259–260bp	FL-6C
	~368–372bp	JOE-6C
	~369–370bp	CXR-6C
Rat	~300bp	FL-6C

9. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **genetic@promega.com**

For questions about GeneMarker® HID software, contact SoftGenetics at: **www.softgenetics.com**

9.A. Amplification and Fragment Detection

This section provides information about general amplification and detection. For questions about amplification of extracted DNA, see Section 9.B. For questions about direct amplification, see Sections 9.C and 9.D.

Symptoms	Causes and Comments
Faint or absent allele peaks	The PowerPlex® Fusion 6C 5X Master Mix was not vortexed well before use. Vortex the 5X Master Mix for 15 seconds before dispensing into the PCR amplification mix.
	An air bubble formed at the bottom of the reaction tube. Use a pipette to remove the air bubble, or centrifuge the reactions briefly before thermal cycling.
	Thermal cycler, plate or tube problems. Review the thermal cycling protocol in Section 4, 11.D or 11.E. We have not tested other reaction tubes, plates or thermal cyclers. Calibrate the thermal cycler heating block if necessary.
	Primer concentration was too low. Use the recommended primer concentration. Vortex the PowerPlex® Fusion 6C 5X Primer Pair Mix for 15 seconds before use.
	Poor capillary electrophoresis injection (WEN ILS 500 peaks also affected). Re-inject the sample. Check the laser power.
	Samples were not denatured completely. Heat-denature samples for the recommended time, then cool on crushed ice or a freezer plate block or in an ice-water bath immediately prior to capillary electrophoresis. Do not cool samples in a thermal cycler set at 4°C, as this may lead to artifacts due to DNA re-annealing.
	Poor-quality formamide was used. Use only Hi-Di™ formamide when analyzing samples.
Faint or absent allele peaks for the positive control reaction	Improper storage of the 2800M Control DNA.

9.A. Amplification and Fragment Detection (continued)

Symptoms	Causes and Comments
Extra peaks visible in one or all color channels	Contamination with another template DNA or previously amplified DNA. Cross-contamination can be a problem. Use aerosol-resistant pipette tips, and change gloves regularly.
	Samples were not denatured completely. Heat denature samples for the recommended time, and cool on crushed ice or a freezer plate block or in an ice-water bath immediately prior to capillary electrophoresis. Do not cool samples in a thermal cycler set at 4°C, as this may lead to artifacts due to DNA re-annealing.
	Double-stranded DNA migrates faster than single-stranded DNA during capillary electrophoresis. Appearance of "shadow" peaks migrating in front of the main peaks, especially if the shadow peaks are separated by the same distance as the main peaks in a heterozygote, can indicate the presence of double-stranded DNA due to incomplete denaturation or post-injection re-annealing.
	Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete addition of the 3´ A residue. • Be sure to perform a 10-minute extension step at 60°C after thermal cycling (Section 4, 11.D or 11.E). • Decrease the amount of template DNA. Using more than the recommended amount of template DNA can result in incomplete adenylation. • Decrease cycle number. • Increase the final extension time.
	CE-related artifacts ("spikes"). Minor voltage changes or urea crystals passing by the laser can cause "spikes" or unexpected peaks. Spikes sometimes appear in one color but often are easily identified by their presence in more than one color. Re-inject samples to confirm.
	Incorrect J6 spectral was active when analyzing samples with the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer. Re-run samples, and confirm that the PowerPlex® 6C J6 spectral is set for J6. See instructions for instrument preparation in Section 5.B.

Symptoms	Causes and Comments
Extra peaks visible in one or all color channels (continued)	Pull-up or bleedthrough. Pull-up can occur when peak heights are too high or if a poor or incorrect matrix is applied to the samples. • Perform a new spectral calibration, and re-run the samples. • Instrument sensitivities can vary. Optimize the injection conditions. See Section 5. • Reboot the Applied Biosystems® 3500 or 3500xL Genetic Analyzer and the instrument's computer. Repeat the spectral calibration. Do not allow borrowing when running the spectral calibration on the Applied Biosystems® 3500 or 3500xL Genetic Analyzer.
	Repeat sample preparation using fresh formamide. Long-term storage of amplified sample in formamide can result in artifacts.
	The CE polymer was beyond its expiration date, or polymer was stored at room temperature for more than one week.
	Maintain instrumentation on a daily or weekly basis, as recommended by the manufacturer.
	Polymer-related artifacts. This system was developed using POP-4® polymer. To use other polymers, optimization and in-house validation are required. The use of POP-7™ polymer can change the migration and sizing location of artifacts compared to that with POP-4® polymer.
Allelic ladder not running the same as samples	Allelic ladder and primer pair mix were not compatible. Ensure that the allelic ladder is from the same kit as the primer pair mix.
	Poor-quality formamide. Use only Hi-Di™ formamide when analyzing samples.
	Be sure the allelic ladder and samples are from the same instrument run.
	Migration of samples changed slightly over the course of a CE run with many samples. This may be due to changes in temperature or the CE column over time. Use a different injection of allelic ladder to determine sizes.
	Poor injection of allelic ladder. Include more than one ladder per instrument run.
	Internal size standard was not assigned correctly. Evaluate the sizing labels on the WEN ILS 500, and correct if necessary.

9.A. Amplification and Fragment Detection (continued)

Symptoms	Causes and Comments
Peak height imbalance	Miscellaneous balance problems. At the first use, thaw the 5X Primer Pair Mix and 5X Master Mix completely. Vortex the 5X Primer Pair Mix and 5X Master Mix for 15 seconds before use; do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after mixing. Calibrate thermal cyclers and pipettes routinely.
	PCR amplification mix prepared in Section 4, 11.D or 11.E was not mixed well. Vortex the PCR amplification mix for 5–10 seconds before dispensing into the reaction tubes or plate.

9.B. Amplification of Extracted DNA

The following information is specific to amplification of extracted DNA. For information about general amplification and detection, see Section 9.A.

Symptoms	Causes and Comments
Faint or absent allele peaks	Impure template DNA. Because a small amount of template is used, this is rarely a problem. Depending on the DNA extraction procedure used and sample source, inhibitors might be present in the DNA sample. Faint or absent peaks may be seen more often when using the maximum template volume or reduced amplification reaction volume.
	Insufficient template. Use the recommended amount of template DNA if available.
	High salt concentration or altered pH. If the DNA template is stored in TE buffer that is not pH 8.0 or contains a higher EDTA concentration, the DNA volume should not exceed 20% of the total reaction volume. Carryover of K^+, Na^+, Mg^{2+} or EDTA from the DNA sample can negatively affect PCR. A change in pH also may affect PCR. Store DNA in TE^{-4} buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA), TE^{-4} buffer with 20µg/ml glycogen or nuclease-free water. Faint or absent peaks may be seen more often when using the maximum template volume or reduced amplification reaction volume.
Extra peaks visible in one or all color channels	Artifacts of STR amplification. Amplification of excess amounts of purified DNA can result in a higher number of artifact peaks. Use the recommended amount of template DNA. See Section 8 for additional information about stutter and artifacts. The amount of template will need to be optimized if you are using reduced reaction volumes.

Symptoms	Causes and Comments
Peak height imbalance	Excessive amount of DNA. Amplification of >1.0ng of template in a 25µl reaction volume can result in an imbalance, with smaller loci showing more product than larger loci. Use less template or fewer cycles.
	Degraded DNA sample. DNA template was degraded, and larger loci showed diminished yield.
	Insufficient template DNA. Use the recommended amount of template DNA if available. Stochastic effects can occur when amplifying low amounts of template.
	Impure template DNA. Inhibitors that may be present in forensic samples can lead to allele dropout or imbalance. Imbalance may be seen more often when using the maximum template volume or a reduced amplification reaction volume.

9.C. Direct Amplification of DNA From Storage Card Punches

The following information is specific to direct amplification of DNA from storage card punches. For additional information about general amplification and detection, see Section 9.A.

Symptoms	Causes and Comments
Faint or absent allele peaks	AmpSolution™ Reagent was not included in 12.5µl reactions. Be sure to include 5X AmpSolution™ Reagent.
	DNA was not accessible on nonlytic material. Pretreat nonFTA materials with PunchSolution™ Reagent to ensure that DNA is liberated from cellular proteins.
	Poor sample deposition. Shedding and collection of donor cells was variable. Increase cycle number.
	Poor sample transfer to storage card or variable sampling from storage card. Take punches from a different portion of the card. Increasing cycle number can improve low peak heights.
	Too much sample in the reaction. Be sure to use the recommended number of punches. Follow the manufacturer's recommendations when depositing sample onto the storage card.
	Active PunchSolution™ Reagent carried over into amplification reactions with nonFTA card punches. Ensure that the heat block reached 70°C and samples were incubated for 30 minutes or until wells are dry. Incubation for shorter time periods may result in incomplete inactivation of the PunchSolution™ Reagent. We have not tested longer incubation times.
	Inactive PunchSolution™ Reagent was used to pretreat nonFTA punches. Thaw the PunchSolution™ Reagent at 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.

9.C. Direct Amplification of DNA From Storage Card Punches (continued)

Symptoms	Causes and Comments
Faint or absent allele peaks in the positive control reaction	Positive control did not amplify. Do not include a blank punch in the positive control reaction. Presence of blank punches may inhibit amplification of 2800M Control DNA.
Extra peaks visible in one or all color channels	Punch was contaminated. Take punches from blank paper between samples.
	Amplification of processed punches with high amounts of DNA can result in artifact peaks due to overamplification, resulting in saturating signal on the CE instrument. Be sure to use the recommended number of punches. Use of a larger punch size or a smaller reaction volume may result in overamplification and signal saturation. If the signal is saturated, repeat the amplification with a smaller punch, a larger reaction volume or reduced cycle number.
	Amplification of excess template for a given cycle number can result in overloading of the capillary upon electrokinetic injection. The presence of excess DNA in the capillary makes it difficult to maintain the DNA in a denatured single-stranded state. Some single-stranded DNA renatures and becomes double-stranded. Double-stranded DNA migrates faster than single-stranded DNA during capillary electrophoresis and appears as "shadow" peaks migrating in front of the main peaks. If this occurs at a heterozygous locus, it is sometimes possible to see two "shadow" peaks that differ in size from one another by approximately the same distance as the single-stranded alleles.
	Artifacts of STR amplification. Direct amplification of >20ng of template can result in a higher number of artifact peaks. Use the recommended punch size and number of punches. Optimize the cycle number. See Section 8 for additional information on stutter and artifacts.
	Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete addition of the 3´ A residue. • Be sure to perform a 10-minute extension step at 60°C after thermal cycling (Section 4, 11.D or 11.E). • Decrease cycle number. • Increase the final extension time.

Symptoms	Causes and Comments
Peak height imbalance	Excessive amount of DNA. Amplification of >20ng of template can result in an imbalance, with smaller loci showing more product than larger loci. • Be sure to use the recommended number of punches. Follow the manufacturer's recommendations when depositing sample onto the card. • Decrease cycle number.
	The cycle number was too high. Decrease the cycle number by one cycle, and repeat the amplification.
	AmpSolution™ Reagent was not included in 12.5µl reactions. Be sure to include AmpSolution™ Reagent in all 12.5µl reactions when amplifying DNA from punches.
	Amplification was inhibited when using more than one storage card punch with blood. Use only one 1.2mm storage card punch with blood.
	DNA was not accessible on nonlytic material. Small loci may amplify preferentially, with large loci dropping out. Pretreat nonFTA materials with PunchSolution™ Reagent to ensure that DNA is liberated from cellular proteins.
	Active PunchSolution™ Reagent carried over into amplification reactions with nonFTA card punches. Larger loci are most susceptible to carryover and will drop out before the smaller loci. • Ensure that the heat block reached 70°C and samples were incubated for 30 minutes or until wells are dry. Incubation for shorter time periods may result in incomplete inactivation of the PunchSolution™ Reagent. • We recommend treating one 1.2mm nonFTA card punch with 10µl of PunchSolution™ Reagent and using one punch per 12.5µl amplification reaction. Reducing the PunchSolution™ Reagent volume may improve results for reactions with reduced amplification volumes. Optimization and validation are required.
	Inactive PunchSolution™ Reagent was used to pretreat nonFTA punches. Thaw the PunchSolution™ Reagent at 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.
Extreme variability in sample-to-sample peak heights	There can be significant individual-to-individual variability in the number of cells on a punch, resulting in peak height variability between samples. The PunchSolution™ Kit maximizes the recovery of amplifiable DNA from nonFTA punches but does not normalize the amount of DNA present.

9.D. Direct Amplification of DNA From Swabs

The following information is specific to direct amplification of DNA from swabs after pretreatment using the SwabSolution™ Kit. For additional information about general amplification and detection, see Section 9.A.

Symptoms	Causes and Comments
Faint or absent allele peaks	Poor sample deposition. Shedding and collection of donor cells was variable. Increase cycle number.
	Inactive SwabSolution™ Reagent. Thaw the SwabSolution™ Reagent completely in a 37°C water bath, and mix by gentle inversion. Store the SwabSolution™ Reagent at 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.
	Active SwabSolution™ Reagent carried over into the amplification reaction. Ensure that the heat block reached 70°C (90°C if using a 2.2ml, Square-Well Deep Well Plate) and samples were incubated for the full 30 minutes. Incubation for shorter time periods may result in incomplete reagent inactivation. Do not use an incubator to incubate tubes or plates; heat transfer is inefficient and will result in poor performance. Use only a heat block to maintain efficient heat transfer. We have tested 60-minute incubation times and observed no difference in performance compared to a 30-minute incubation.
	DNA was not accessible on nonlytic material. Pretreat swabs with SwabSolution™ Reagent to ensure that DNA is liberated from cellular proteins.
Faint or absent peaks for the positive control reaction	If the positive control reaction failed to amplify, check to make sure that the correct amount of 2800M Control DNA was added to the reaction. Due to the reduced cycle numbers used with swab extracts, it is necessary to increase the mass of 2800M Control DNA to obtain a profile. We recommend 10ng of 2800M Control DNA per 12.5µl amplification reaction. This mass of DNA should be reduced if cycle number is increased and decreased if the cycle number is increased. Increase or decrease by twofold the mass of 2800M Control DNA f or every one-cycle decrease or increase, respectively.
	Improper storage of the 2800M Control DNA.

Symptoms	Causes and Comments
Extra peaks visible in one or all color channels	Swab extract was contaminated. Assemble a reaction containing the swab extract prepared from a blank swab, or assemble a reaction where the SwabSolution™ Reagent is processed and incubated as a blank without a swab.
	Artifacts of STR amplification. Amplification of swab extracts with high DNA concentrations can result in artifact peaks due to overamplification, resulting in saturated signal on the CE instrument. We recommend 2µl of swab extract per reaction. Using more than 2µl may result in overamplification and signal saturation. If signal is saturated, repeat amplification with less swab extract or reduced cycle number.
	Amplification of excess template for a given cycle number resulted in overloading of the capillary upon electrokinetic injection. Excess DNA in the capillary is difficult to maintain in a denatured single-stranded state. Some single-stranded DNA renatures and becomes double-stranded. Double-stranded DNA migrates faster than single-stranded DNA during capillary electrophoresis and appears as "shadow" peaks migrating in front of the main peaks. If this occurs at a heterozygous locus it is possible to observe the presence of two "shadow" peaks that differ in size by approximately the same distance as the single-stranded alleles.
	Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete addition of the 3´ A residue. • Be sure to perform a 10-minute extension step at 60°C after thermal cycling (Section 4, 11.D or 11.E) • Use 2µl of swab extract in a PowerPlex® Fusion 6C reaction. A larger volume of swab extract may contain more than the recommended amount of DNA template, resulting in incomplete adenylation. • Decrease cycle number. • Increase the final extension time.

9.D. Direct Amplification of DNA From Swabs (continued)

Symptoms	Causes and Comments
Peak height imbalance	Excess DNA in the amplification reaction can result in locus-to-locus imbalance within a dye channel such that the peak heights at the smaller loci are greater than those at the larger loci (ski-slope effect). Use less swab extract, or reduce cycle number.
	The cycle number was too high. Decrease cycle number by one cycle, and repeat the amplification.
	Active SwabSolution™ Reagent carried over from swab extracts into the amplification reaction. Larger loci are most susceptible to reagent carryover and will drop out before the smaller loci. Ensure that the heat block reached 70°C (90°C if using 2.2ml, Square-Well Deep Well Plates) and samples were incubated for the full 30 minutes. Incubation for shorter time periods may result in incomplete reagent inactivation. Do not use an incubator to incubate tubes or plates; heat transfer is inefficient and will result in poor performance. Use only a heat block to maintain efficient heat transfer.
	Inactive SwabSolution™ Reagent. Thaw the SwabSolution™ Reagent completely in a 37°C water bath, and mix by gentle inversion. Store the SwabSolution™ Reagent at 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not re-freeze, as this may reduce activity.
	DNA was not accessible on nonlytic material. Small loci may amplify preferentially, with large loci dropping out. Pretreat swabs with SwabSolution™ Reagent to ensure that DNA is liberated from cellular proteins.
Extreme variability in sample-to-sample peak heights	There can be significant individual-to-individual variability in cell deposition onto buccal swabs. This will appear as variability in peak heights between swab extracts. The extraction process maximizes recovery of amplifiable DNA from buccal swabs but does not normalize the amount of DNA present. If variability is extreme, quantitate the DNA using a fluorescence-based double-stranded DNA quantitation method or qPCR-based quantitation method. The quantitation values can be used to normalize input template amounts to minimize variation in signal intensity.

9.E. GeneMapper® *ID*-X Software

Symptoms	Causes and Comments
Stutter peaks not filtered	Stutter text file was not imported into the Panel Manager when the panels and bins text files were imported.
	Be sure that the "Use marker-specific stutter ratio and distance if available" box is checked. If the "Use marker-specific stutter ratio and distance if available" box is not checked, stutter distance must be defined in the Analysis Method Allele tab.
Samples in the project not analyzed	The Analysis Requirement Summary window was not active, and there was an analysis requirement that was not met. Turn on Analysis Requirement Summary in the Options menu, and correct the necessary analysis requirements to continue analysis.
Edits in label edit viewer cannot be viewed	To view edits made to a project, the project first must be saved. Close the plot view window, return to the main GeneMapper® *ID*-X page and save the project. Display the plot window again, then view the label edit table.
Marker header bar for some loci are gray	When an edit is made to a locus, the quality flags and marker header bar automatically change to gray. To change the GQ and marker header bar for a locus to green, override the GQ in the plot window.
Alleles not called	To analyze samples with GeneMapper® *ID*-X software, at least one allelic ladder must be defined.
	An insufficient number of WEN ILS 500 fragments was defined. Be sure to define at least two WEN ILS 500 fragments smaller than the smallest sample peak and at least two WEN ILS 500 fragments larger than the largest sample peak. In this instance, the allelic ladder would have failed the allelic ladder quality check.
	Run was too short, and larger peaks in ILS were not captured. Not all WEN ILS 500 peaks defined in the size standard were detected during the run. • Create a new size standard using the internal lane standard fragments present in the sample. • Re-run samples using a longer run time.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.

9.E. GeneMapper® *ID*-X Software (continued)

Symptoms	Causes and Comments
Off-ladder alleles	An allelic ladder from a different run than the samples was used. Re-analyze samples with an allelic ladder from the same run.
	The GeneMapper® *ID*-X software requires that the allelic ladder be imported from the same folder as the sample. Be sure that the allelic ladder is in the same folder as the sample. Create a new project and re-analyze, as described in Section 6.D or 6.E.
	Panels text file selected for analysis was incorrect for the STR system used. Assign correct panels text file that corresponds to the STR system used for amplification.
	The allelic ladder was not identified as an allelic ladder in the Sample Type column.
	The internal lane standard was not properly identified in the sample. Manually redefine the sizes of the size standard fragments in the sample.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.
	Incorrect polymer was used. Use of a polymer other than POP-4® polymer may change migration of the fragments. Alleles may migrate outside of the panel range established using POP-4® polymer.
Size standard not called correctly	Starting data point was incorrect for the partial range chosen in Section 6.D or 6.E. Adjust the starting data point in the analysis method. Alternatively, use a full range for the analysis.
	Extra peaks in size standard. Open the Size Match Editor. Highlight the extra peak, select "Edit" and select "delete size label". Select "auto adjust sizes".
	Run was too short, and larger peaks in ILS were not captured. Not all WEN ILS 500 peaks defined in the size standard were detected during the run. • Create a new size standard using the internal lane standard fragments present in the sample. • Re-run samples using a longer run time.
Peaks in size standard missing	If peaks are below threshold, decrease the peak amplitude threshold in the analysis method for the orange channel to include peaks or increase the volume of WEN ILS 500 used in Section 5.
	If peaks are low-quality, redefine the size standard for the sample to skip these peaks.
Significantly raised baseline	Poor spectral calibration. Perform a new spectral calibration, and re-run the samples.
	Incorrect dye set was used.

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11. Appendix

11.A. Advantages of Using the Loci in the PowerPlex® Fusion 6C System

A single PowerPlex® Fusion 6C System reaction amplifies all core loci required for US expanded CODIS and European databases (Tables 7 and 8). The male-specific DYS391 locus is included to identify null Y results for Amelogenin. Additionally, two rapidly mutating Y-STR loci are included in the system. Table 9 lists the PowerPlex® Fusion 6C System alleles amplified from commonly available standard DNA templates.

We have carefully selected primers to avoid or minimize artifacts, including those associated with DNA polymerases, such as repeat slippage and terminal nucleotide addition (14,15). Repeat slippage, sometimes called “n–4 peaks”, “stutter” or “shadow peaks”, is due to the loss of a repeat unit during DNA amplification, somatic variation within the DNA or both. The amount of this artifact observed depends primarily on the locus and the DNA sequence being amplified.

Terminal nucleotide addition (16,17) occurs when a thermostable nonproofeading DNA polymerase adds a nucleotide, generally adenine, to the 3´ ends of amplified DNA fragments in a template-independent manner. The efficiency with which this occurs varies with different primer sequences. Thus, an artifact peak one base shorter than expected (i.e., missing the terminal addition) is sometimes seen. We have modified primer sequences and added a final extension step at 60°C (18) to the amplification protocols to provide conditions for essentially complete terminal nucleotide addition when recommended amounts of template DNA are used.

Table 7. The PowerPlex® Fusion 6C System Locus-Specific Information.

STR Locus	Label	Chromosomal Location[1]	Repeat Sequence[2] 5´→3´
Amelogenin[3]	FL-6C	Xp22.1–22.3 and Y	NA
D3S1358	FL-6C	3p21.31 (45.557Mb)	TCTA Complex
D1S1656	FL-6C	1q42 (228.972Mb)	TAGA Complex
D2S441	FL-6C	2p14 (68.214Mb)	TCTA
D10S1248	FL-6C	10q26.3 (130.567Mb)	GGAA
D13S317	FL-6C	13q31.1 (81.62Mb)	TATC
Penta E	FL-6C	15q26.2 (95.175Mb)	AAAGA
D16S539	JOE-6C	16q24.1 (84.944Mb)	GATA
D18S51	JOE-6C	18q21.33 (59.1Mb)	AGAA (19)
D2S1338	JOE-6C	2q35 (218.705Mb)	TGCC/TTCC
CSF1PO	JOE-6C	5q33.1 (149.436Mb)	AGAT
Penta D	JOE-6C	21q22.3 (43.88Mb)	AAAGA
TH01	TMR-6C	11p15.5 (2.149Mb)	AATG (19)
vWA	TMR-6C	12p13.31 (5.963Mb)	TCTA Complex (19)
D21S11	TMR-6C	21q21.1 (19.476Mb)	TCTA Complex (19)
D7S820	TMR-6C	7q21.11 (83.433Mb)	GATA
D5S818	TMR-6C	5q23.2 (123.139Mb)	AGAT
TPOX	TMR-6C	2p25.3 (1.472Mb)	AATG
D8S1179	CXR-6C	8q24.13 (125.976Mb)	TCTA Complex (19)
D12S391	CXR-6C	12p12 (12.341Mb)	AGAT/AGAC Complex
D19S433	CXR-6C	19q12 (35.109Mb)	AAGG Complex
SE33	CXR-6C	6q14 (89.043Mb)	AAAG Complex
D22S1045	CXR-6C	22q12.3 (35.779Mb)	ATT
DYS391	TOM-6C	Y	TCTA
FGA	TOM-6C	4q28 (155.866Mb)	TTTC Complex (19)
DYS576	TOM-6C	Y	AAAG
DYS570	TOM-6C	Y	TTTC

[1]Information about the chromosomal location of these loci can be found in references 20, 21 and 22 and at: **www.cstl.nist.gov/biotech/strbase/chrom.htm**

[2]The August 1997 report (23,24) of the DNA Commission of the International Society for Forensic Haemogenetics (ISFH) states, "1) for STR loci within coding genes, the coding strand shall be used and the repeat sequence motif defined using the first possible 5´ nucleotide of a repeat motif; and 2) for STR loci not associated with a coding gene, the first database entry or original literature description shall be used".

[3]Amelogenin is not an STR but displays an 89-base, X-specific band and a 95-base, Y-specific band.

NA = Not applicable

Promega

Table 8. The PowerPlex® Fusion 6C System Allelic Ladder Information.

STR Locus	Label	Size Range of Allelic Ladder Components[1,2] (bases)	Repeat Numbers of Allelic Ladder Components[3]
Amelogenin	FL-6C	89, 95	X, Y
D3S1358	FL-6C	103–147	9–20
D1S1656	FL-6C	161–208	9–14, 14.3, 15, 15.3, 16, 16.3, 17, 17.3, 18, 18.3, 19, 19.3, 20.3
D2S441	FL-6C	216–252	8–11, 11.3, 12–17
D10S1248	FL-6C	260–284	8–19
D13S317	FL-6C	308–358	5–17
Penta E	FL-6C	371–471	5–25
D16S539	JOE-6C	84–132	4–16
D18S51	JOE-6C	134–214	7–10, 10.2, 11–13, 13.2, 14–27
D2S1338	JOE-6C	224–296	10, 12, 14–28
CSF1PO	JOE-6C	318–362	5–16
Penta D	JOE-6C	377–450	2.2, 3.2, 5–17
TH01	TMR-6C	72–115	3–9, 9.3, 10–11, 13.3
vWA	TMR-6C	127–183	10–24
D21S11	TMR-6C	203–259	24, 24.2, 25, 25.2, 26–28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36–38
D7S820	TMR-6C	269–313	5–16
D5S818	TMR-6C	321–369	6–18
TPOX	TMR-6C	393–441	4–16
D8S1179	CXR-6C	76–124	7–19
D12S391	CXR-6C	133–185	14–17, 17.3, 18, 18.3, 19–27
D19S433	CXR-6C	193–245	5.2, 6.2, 8–12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2, 18, 18.2
SE33	CXR-6C	270–408	4.2, 6.3, 8–20, 20.2, 21, 21.2, 22, 22.2, 23.2, 24.2, 25.2, 26.2, 27.2, 28.2, 29.2, 30.2, 31.2, 32.2, 33.2, 34.2, 35–37, 39
D22S1045	CXR-6C	431–470	7–20
DYS391	TOM-6C	86–130	5–16
FGA	TOM-6C	143–289	14–18, 18.2, 19, 19.2, 20, 20.2, 21, 21.2, 22, 22.2, 23, 23.2, 24, 24.2, 25, 25.2, 26–30, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 48.2, 50.2
DYS576	TOM-6C	308–356	11–23
DYS570	TOM-6C	393–453	10–25

[1]The length of each allele in the allelic ladder has been confirmed by sequence analysis.

[2]When using an internal lane standard, such as the WEN Internal Lane Standard 500, the calculated sizes of allelic ladder components may differ from those listed. This occurs because different sequences in allelic ladder and ILS components may cause differences in migration. The dye label and linker also affect migration of alleles.

[3]For a current list of microvariants, see the Variant Allele Report published at the U.S. National Institute of Standards and Technology (NIST) web site at: **www.cstl.nist.gov/div831/strbase/**

Table 9. The PowerPlex® Fusion 6C System Allele Determinations in Commonly Available Standard DNA Templates.

	Standard DNA Templates[1]		
STR Locus	**2800M**	**9947A**	**9948**
Amelogenin	X, Y	X, X	X, Y
D3S1358	17, 18	14, 15	15, 17
D1S1656	12, 13	18.3, 18.3	14, 17
D2S441	10, 14	10, 14	11, 12
D10S1248	13, 15	13, 15	12, 15
D13S317	9, 11	11, 11	11, 11
Penta E	7, 14	12, 13	11, 11
D16S539	9, 13	11, 12	11, 11
D18S51	16, 18	15, 19	15, 18
D2S1338	22, 25	19, 23	23, 23
CSF1PO	12, 12	10, 12	10, 11, 12[2]
Penta D	12, 13	12, 12	8, 12
TH01	6, 9.3	8, 9.3	6, 9.3
vWA	16, 19	17, 18	17, 17
D21S11	29, 31.2	30, 30	29, 30
D7S820	8, 11	10, 11	11, 11
D5S818	12, 12	11, 11	11, 13
TPOX	11, 11	8, 8	8, 9
D8S1179	14, 15	13, 13	12, 13
D12S391	18, 23	18, 20	18, 24
D19S433	13, 14	14, 15	13, 14
SE33	15, 16	19, 29.2	23.2, 26.2
D22S1045	16, 16	11, 14	16, 18
DYS391	10	–	10
FGA	20, 23	23, 24	24, 26
DYS576	18	–	16
DYS570	17	–	18

[1]Information on strains 9947A and 9948 is available online at: **http://ccr.coriell.org/Sections/Search/Sample_Detail.aspx?Ref=GM09947** and **http://ccr.coriell.org/Sections/Search/Sample_Detail.aspx?Ref=GM09948**

Information about the use of 9947A and 9948 DNA as standard DNA templates can be found in reference 25.

[2]The relative peak heights for these three alleles will differ. Allele 12 may not be called.

11.B. DNA Extraction and Quantitation Methods and Automation Support

Promega offers a wide variety of reagents and automated methods for sample preparation, DNA purification and DNA quantitation prior to STR amplification.

For analysis of database, reference and other single-source samples, we recommend direct amplification of DNA from FTA® card punches or direct amplification of DNA from swabs and nonFTA punches following a preprocessing step with the SwabSolution™ Kit or PunchSolution™ Kit, respectively. The SwabSolution™ Kit (Cat.# DC8271) contains reagents for rapid DNA preparation from buccal swabs prior to amplification. The procedure lyses cells contained on the swab head and releases into solution sufficient DNA for STR amplification. A small volume of the final swab extract is added to the PowerPlex® reaction. The PunchSolution™ Kit is used to process punches from nonFTA storage cards containing blood or buccal samples prior to direct amplification.

For casework or samples that require DNA purification, we recommend the DNA IQ™ System (Cat.# DC6700), which is a DNA isolation system designed specifically for forensic samples (26). This system uses paramagnetic particles to prepare clean samples for STR analysis easily and efficiently and can be used to extract DNA from stains or liquid samples, such as blood or solutions. The DNA IQ™ System eliminates PCR inhibitors and contaminants frequently encountered in casework samples. In additional, DNA has been isolated from casework samples such as tissue, differentially separated sexual assault samples and stains on support materials. The DNA IQ™ System has been tested with PowerPlex® Systems to ensure a streamlined process.

For applications requiring human-specific DNA quantification, the Plexor® HY System (Cat.# DC1000) was developed (27). This qPCR-based method provides total human and male-specific DNA quantification in one reaction. Additionally, the Plexor® HY System provides a post-amplification melt analysis to confirm positive results and an Internal PCR Control (IPC) to confirm negative results. Additional ordering information is available in Section 11.G.

For information about automation of Promega chemistries on automated workstations using Identity Automation™ solutions, contact your local Promega Branch Office or Distributor (contact information available at: **www.promega.com/support/worldwide-contacts/**), e-mail: genetic@promega.com or visit: **www.promega.com/idautomation/**

11.C. The WEN Internal Lane Standard 500

The WEN Internal Lane Standard 500 contains 21 DNA fragments of 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 and 500 bases in length (Figure 29). Each fragment is labeled with WEN-6C dye and can be detected separately (as a sixth color) in the presence of PowerPlex® Fusion 6C-amplified material. The WEN ILS 500 is designed for use in each CE injection to increase precision in analyses when using the PowerPlex® Fusion 6C System. Protocols to prepare and use this internal lane standard are provided in Section 5.

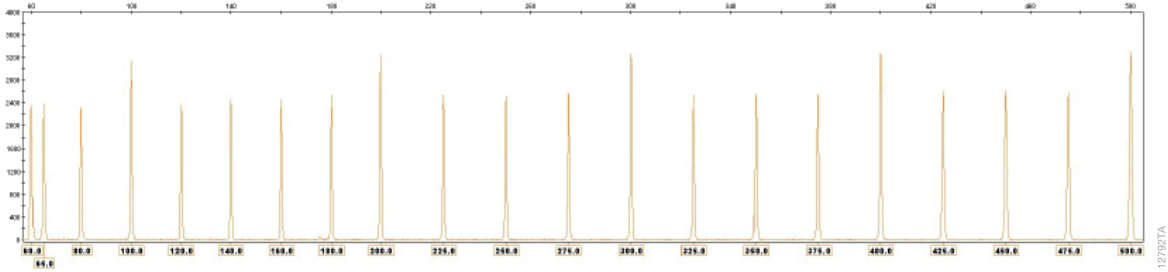

Figure 29. WEN Internal Lane Standard 500. An electropherogram showing the WEN Internal Lane Standard 500 fragments.

11.D. Direct Amplification of DNA from Storage Card Punches in a 25µl Reaction Volume

Depending on the procedure used to prepare storage cards, high amounts of cells may be present in each punch. In these cases, performing direct amplification in 25µl reaction volumes may provide better results.

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700, 96-Well, with a gold-plated or silver-plated sample block or Veriti® 96-Well Thermal Cycler (Applied Biosystems)
- centrifuge compatible with a 96-well plate
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- PunchSolution™ Kit (Cat.# DC9271) for nonFTA card punches
- 1.2mm Harris Micro-Punch or equivalent manual punch and cutting mat or automated punch system

This section contains a protocol for direct amplification of DNA from storage card punches in 25µl reaction volumes using the PowerPlex® Fusion 6C System and GeneAmp® PCR System 9700 or Veriti® 96-Well Thermal Cycler. The PowerPlex® Fusion 6C System contains sufficient reagents for 100 direct-amplification reactions of 12.5µl each; when performing 25µl reactions, the system contains sufficient reagents for 50 reactions.

The PowerPlex® Fusion 6C System is compatible with the GeneAmp® PCR System 9700 thermal cycler with a silver-plated or gold-plated sample block and the Veriti® 96-Well Thermal Cycler. This system has not been tested with the Veriti® 96-Well Fast Thermal Cycler or the GeneAmp® PCR System 9700 with an aluminum block.

For 25µl amplification reactions with FTA® cards, we recommend amplifying one or two 1.2mm punches of a storage card containing buccal cells or one 1.2mm punch of a storage card containing whole blood. For nonFTA cards, we recommend amplifying one 1.2mm punch of a storage card containing buccal cells or whole blood.

11.D. Direct Amplification of DNA from Storage Card Punches in 25µl Reaction Volume (continued)

Note: You will need to optimize and validate the number of storage card punches per reaction in your laboratory. See the PCR optimization recommendations at the end of the section.

FTA®-based sample types include:

- Buccal cells collected on FTA® cards with Whatman EasiCollect™ or Fitzco Sampact™ devices
- Buccal cells collected with swabs transferred to FTA® or Indicating FTA® cards
- Liquid blood (from collection or storage Vacutainer® tubes or finger sticks) spotted onto FTA® cards

NonFTA sample types include:

- Buccal samples on Bode Buccal DNA Collector™ devices
- Blood and buccal samples on nonFTA card punches (e.g., S&S 903)

Pretreat nonFTA sample types with the PunchSolution™ Kit (Cat.# DC9271) to lyse nonFTA samples before adding the PCR amplification mix. For more information, see the *PunchSolution™ Kit Technical Manual* #TMD038. Failure to pretreat these samples may result in incomplete profiles.

Use a manual punch tool with a 1.2mm tip to manually create sample disks from a storage card. Place tip near the center of the sample spot, and with a twisting or pressing action, cut a 1.2mm sample disk. Use the plunger to eject the disk into the appropriate well of a reaction plate.

Automated punchers also can be used to create sample disks. Refer to the user's guide for your instrument for assistance with generating 1.2mm disks, technical advice and troubleshooting information.

Note: Static may be problematic when adding a punch to a well. For FTA® card punches, adding PCR amplification mix to the well before adding the punch may help alleviate static problems. For nonFTA card punches, adding PunchSolution™ Reagent to the well before adding the punch during pretreatment may help alleviate static problems.

Amplification Setup

1. At the first use, thaw the PowerPlex® Fusion 6C 5X Master Mix, PowerPlex® Fusion 6C 5X Primer Pair Mix and Water, Amplification Grade, completely. After the first use, store the reagents at 2–10°C.

   Note: Centrifuge tubes briefly to bring contents to the bottom, then vortex reagents for 15 seconds before each use. Do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.

3. Use a clean MicroAmp® plate for reaction assembly, and label appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label appropriately.

4. Add the final volume of each reagent listed in Table 10 to a sterile tube.

Table 10. PCR Amplification Mix for Direct Amplification of DNA from Storage Card Punches Using a 25µl Reaction Volume.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	15.0µl	×		=	
PowerPlex® Fusion 6C 5X Master Mix	5.0µl	×		=	
PowerPlex® Fusion 6C 5X Primer Pair Mix	5.0µl	×		=	
total reaction volume	25µl				

[1]Add Water, Amplification Grade, to the tube first, then add PowerPlex® Fusion 6C 5X Master Mix and PowerPlex® Fusion 6C 5X Primer Pair Mix. For FTA® card punches, the template DNA will be added at Step 6.

5. Vortex the PCR amplification mix for 5–10 seconds, then pipet 25µl of PCR amplification mix into each reaction well.

! Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or locus-to-locus imbalance.

6. For FTA® storage cards, add one or two 1.2mm punches from a card containing buccal cells or one 1.2mm punch from a card containing whole blood to the appropriate wells of the reaction plate. For nonFTA card punches, add the PCR amplification mix to the PunchSolution™ Reagent-treated punch.

   Note: It also is acceptable to add the FTA® card punch first, then add the PCR amplification mix.

7. For the positive amplification control, vortex the tube of 2800M Control DNA, then add 1µl (10ng) to a reaction well containing 25µl of PCR amplification mix.

   Notes:

   1. Do not include blank storage card punches in the positive control reactions.
   2. Optimization of the amount of 2800M Control DNA may be required, depending on cycling conditions and laboratory preferences.

8. Reserve a well containing PCR amplification mix as a negative amplification control.

   Note: An additional negative control with a blank punch may be performed to detect contamination from the storage card or punch device.

9. Seal or cap the plate, or close the tubes. Briefly centrifuge the plate to bring storage card punches to the bottom of the wells and remove air bubbles.

11.D. Direct Amplification of DNA from Storage Card Punches in 25µl Reaction Volume (continued)

Thermal Cycling

Amplification and detection instrumentation may vary. You will need to optimize protocols including the number of storage card punches, cycle number (24–27 cycles), injection time and loading volume for your laboratory instrumentation. Testing at Promega shows that 26 cycles works well for a variety of sample types. Buccal samples may require more amplification cycles than blood samples. NonFTA card punches may require fewer amplification cycles than FTA® punches. Cycle number should be optimized in each laboratory for each sample type.

1. Place the MicroAmp® plate in the thermal cycler.
2. Select and run the recommended protocol, which is provided below and in Figure 30. The total cycling time is approximately 1 hour.

   Notes:

   1. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.
   2. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a silver-plated or gold-plated sample block. The ramp speed is set after the thermal cycling run is started. When the Select Method Options screen appears, select "Max" for the ramp speed and enter the reaction volume.

Thermal Cycling Protocol
96°C for 1 minute, then:
96°C for 5 seconds 60°C for 1 minute for 26 cycles, then:
60°C for 10 minutes
4°C soak

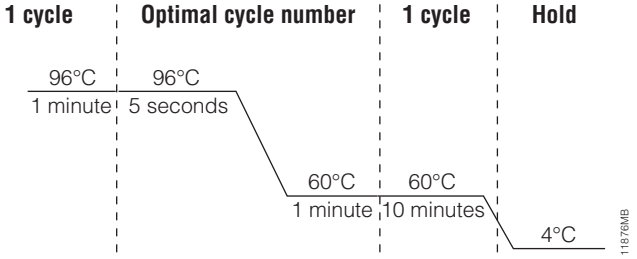

Figure 30. Thermal cycling protocol for the GeneAmp® PCR System 9700 and Veriti® 96-Well Thermal Cycler.

3. After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at –20°C in a light-protected box.

   Note: Long-term storage of amplified samples at 4°C or higher may produce artifacts.

PCR Optimization

Cycle number should be optimized based on the results of an initial experiment to determine the sensitivity with your collection method, sample types, number of punches and instrumentation.

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.
2. Depending on your preferred protocol, place one or two 1.2mm FTA® storage card punches containing buccal cells, one 1.2mm FTA® storage card punch containing whole blood or one 1.2mm punch of a nonFTA storage card containing buccal cells or whole blood in each well of a reaction plate. Be sure to pretreat nonFTA samples with the PunchSolution™ Kit (Cat.# DC9271).
3. Prepare four identical reaction plates with punches from the same samples.
4. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (24–27 cycles).
5. Following amplification, use your laboratory's validated separation and detection protocols to determine the optimal cycle number for the sample type and number of storage card punches.

11.E. Direct Amplification of DNA from Swabs in a 25µl Reaction Volume

Depending on the procedure used to prepare the swabs, high amounts of cells may be present on each swab. In these cases, performing direct amplification in 25µl reaction volumes may provide better results.

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700, 96-Well, with a gold-plated or silver-plated sample block or Veriti® 96-Well Thermal Cycler (Applied Biosystems)
- centrifuge compatible with a 96-well plate
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- SwabSolution™ Kit (Cat.# DC8271)

This section contains a protocol for amplifying DNA from swab extracts in 25µl reaction volumes using the PowerPlex® Fusion 6C System and GeneAmp® PCR System 9700 or Veriti® 96-Well Thermal Cycler. The PowerPlex® Fusion 6C System contains sufficient reagents for 100 direct-amplification reactions of 12.5µl each; when performing 25µl reactions, the system contains sufficient reagents for 50 reactions.

The PowerPlex® Fusion 6C System is compatible with the GeneAmp® PCR System 9700 with a silver-plated or gold-plated sample block and the Veriti® 96-Well Thermal Cycler. This system has not been tested with the Veriti® 96-Well Fast Thermal Cycler or the GeneAmp® PCR System 9700 with an aluminum block.

Pretreat OmniSwab™ (GE Healthcare) or cotton swabs with the SwabSolution™ Kit (Cat.# DC8271) as described in the *SwabSolution™ Kit Technical Manual* #TMD037 to generate a swab extract.

11.E. Direct Amplification of DNA from Swabs in a 25µl Reaction Volume (continued)

Amplification Setup

1. At the first use, thaw the PowerPlex® Fusion 6C 5X Master Mix, PowerPlex® Fusion 6C 5X Primer Pair Mix and Water, Amplification Grade, completely. After the first use, store the reagents at 2–10°C.

   Note: Centrifuge tubes briefly to bring contents to the bottom, then vortex reagents for 15 seconds before each use. Do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.

3. Use a clean MicroAmp® plate for reaction assembly, and label appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label appropriately.

4. Add the final volume of each reagent listed in Table 11 to a sterile tube.

Table 11. PCR Amplification Mix for Direct Amplification of DNA from Swabs Using a 25µl Reaction Volume.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	13µl	×		=	
PowerPlex® Fusion 6C 5X Master Mix	5.0µl	×		=	
PowerPlex® Fusion 6C 5X Primer Pair Mix	5.0µl	×		=	
swab extract	2.0µl				
total reaction volume	25µl				

[1]Add Water, Amplification Grade, to the tube first, then add PowerPlex® Fusion 6C 5X Master Mix and PowerPlex® Fusion 6C 5X Primer Pair Mix. The swab extract will be added at Step 6.

5. Vortex the PCR amplification mix for 5–10 seconds, then pipet 23µl of PCR amplification mix into each reaction well.

   Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or locus-to-locus imbalance.

6. Pipet 2.0µl of swab extract for each sample into the appropriate well of the reaction plate.

7. For the positive amplification control, vortex the tube of 2800M Control DNA, then dilute an aliquot to 5.0ng/µl. Add 2µl (10ng) to a reaction well containing 23µl of PCR amplification mix.

   Note: Optimization of the amount of 2800M Control DNA may be required, depending on thermal cycling conditions and laboratory preferences.

8. For the negative amplification control, pipet 2.0µl of Water, Amplification Grade, or TE^{-4} buffer instead of swab extract into a reaction well containing PCR amplification mix.

   Note: Additional negative controls can be included. Assemble a reaction containing the swab extract prepared from a blank swab, or assemble a reaction where the SwabSolution™ Reagent is processed as a blank without a swab.

9. Seal or cap the plate, or close the tubes. **Optional:** Briefly centrifuge the plate to bring contents to the bottom of the wells and remove any air bubbles.

Thermal Cycling

Amplification and detection instrumentation may vary. You will need to optimize protocols including the amount of template DNA, cycle number (25–28 cycles), injection time and loading volume for your laboratory instrumentation. Testing at Promega shows that 26 cycles works well for a variety of sample types. Cycle number will need to be optimized in each laboratory for each sample type.

1. Place the MicroAmp® plate in the thermal cycler.
2. Select and run the recommended protocol, which is provided below and in Figure 31. The total cycling time is approximately 1 hour.

   Notes:

   1. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.
   2. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a silver-plated or gold-plated sample block. The ramp speed is set after the thermal cycling run is started. When the Select Method Options screen appears, select "Max" for the ramp speed and enter the reaction volume.

Thermal Cycling Protocol
96°C for 1 minute, then:
96°C for 5 seconds 60°C for 1 minute for 26 cycles, then:
60°C for 10 minutes
4°C soak

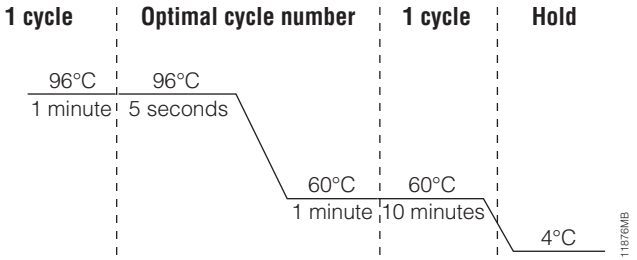

Figure 31. Thermal cycling protocol for the GeneAmp® PCR System 9700 and Veriti® 96-Well Thermal Cycler.

3. After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at –20°C in a light-protected box.

   Note: Long-term storage of amplified samples at 4°C or higher may produce artifacts.

11.E. Direct Amplification of DNA from Swabs in a 25µl Reaction Volume (continued)

PCR Optimization

Cycle number should be optimized based on the results of an initial experiment to determine the sensitivity with your collection method, sample types and instrumentation.

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.
2. Prepare four identical reaction plates with aliquots of the same swab extracts.
3. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (25–28 cycles).
4. Following amplification, use your laboratory's validated separation and detection protocols to determine the optimal cycle number for the sample type.

11.F. Composition of Buffers and Solutions

TE^{-4} buffer (10mM Tris-HCl, 0.1mM EDTA [pH 8.0])

1.21g	Tris base
0.037g	EDTA ($Na_2EDTA \cdot 2H_2O$)

Dissolve Tris base and EDTA in 900ml of deionized water. Adjust to pH 8.0 with HCl. Bring the final volume to 1 liter with deionized water.

TE^{-4} buffer with 20µg/ml glycogen

1.21g	Tris base
0.037g	EDTA ($Na_2EDTA \cdot 2H_2O$)
20µg/ml	glycogen

Dissolve Tris base and EDTA in 900ml of deionized water. Adjust to pH 8.0 with HCl. Add glycogen. Bring the final volume to 1 liter with deionized water.

11.G. Related Products

STR Systems

Product	Size	Cat.#
PowerPlex® Fusion System	200 reactions	DC2402
	800 reactions	DC2408
PowerPlex® Y23 System	50 reactions	DC2305
	200 reactions	DC2320
PowerPlex® 21 System	200 reactions	DC8902
	4 × 200 reactions	DC8942
PowerPlex® 18D System	200 reactions	DC1802
	800 reactions	DC1808
PowerPlex® ESX 16 Fast System	100 reactions	DC1611
	400 reactions	DC1610
PowerPlex® ESX 17 Fast System	100 reactions	DC1711
	400 reactions	DC1710
PowerPlex® ESI 16 Fast System	100 reactions	DC1621
	400 reactions	DC1620
PowerPlex® ESI 17 Fast System	100 reactions	DC1721
	400 reactions	DC1720
PowerPlex® 16 HS System	100 reactions	DC2101
	400 reactions	DC2100
PowerPlex® CS7 System	100 reactions	DC6613

Not for Medical Diagnostic Use.

Accessory Components

Product	Size	Cat.#
PowerPlex® 6C Matrix Standard	5 preps	DG4900
WEN Internal Lane Standard 500	200µl	DG5001
PunchSolution™ Kit	100 preps	DC9271
SwabSolution™ Kit	100 preps	DC8271
5X AmpSolution™ Reagent	100 preps	DM1231
2800M Control DNA (10ng/µl)	25µl	DD7101
2800M Control DNA (0.25ng/µl)	500µl	DD7251
Water, Amplification Grade	6,250µl (5 × 1,250µl)	DW0991

Not for Medical Diagnostic Use.

11.G. Related Products (continued)

Sample Preparation and DNA Quantification Systems

Product	Size	Cat.#
DNA IQ™ System	100 reactions	DC6701
	400 reactions	DC6700
Plexor® HY System*	200 reactions	DC1001
	800 reactions	DC1000

*Not for Medical Diagnostic Use.

[a]Patents Pending.

[b]U.S. Pat. No. 6,242,235, Australian Pat. No. 761757, Canadian Pat. No. 2,335,153, Chinese Pat. No. ZL99808861.7, Hong Kong Pat. No. HK 1040262, Japanese Pat. No. 3673175, European Pat. No. 1088060 and other patents pending.

[c]U.S. Pat. No. 6,479,235, Australian Pat. No. 724531, Canadian Pat. No. 2,251,793, Korean Pat. No. 290332, Singapore Pat. No. 57050, Japanese Pat. Nos. 3602142 and 4034293, Chinese Pat. Nos. ZL99813729.4 and ZL97194967.0, European Pat. No. 0960207 and other patents pending.

[d]U.S. Pat. No 6,238,863, European Pat. No. 1058727, Chinese Pat. No. ZL99802696.4, Japanese Pat. No. 4494630 and other patents pending.

[e]STR loci are the subject of U.S. Pat. No. RE 37,984, German Pat. No. DE 38 34 636 C2 and other patents issued to the Max-Planck-Gesellschaft zur Förderung der Wissenschaften, e.V., Germany.

[f]Allele sequences for one or more of the loci vWA, FGA, D8S1179, D21S11 and D18S51 in allelic ladder mixtures is licensed under U.S. Pat. Nos. 7,087,380, 7,645,580, Australia Pat. No. 2003200444 and corresponding patent claims outside the US.

[g]TMR-6C, CXR-6C, TOM-6C and WEN-6C dyes are proprietary.

[h]This product or portions thereof is manufactured and sold under license from GE Healthcare under Australia Pat. No. 692230, Austria Pat. No. E236994, Belgium Pat. No. 0743987, Canada Pat. No. 2231475, EP Pat. Nos. 0743987 and 0851867, France Pat. Nos. 0743987 and 0851867, Germany Pat. Nos. 19581489, 69530286.8 and 0851867, Italy Pat. Nos. 0743987 and 0851867, Japan Pat. No. 3066984, Liechtenstein Pat. Nos. 0743987 and 0851867, Netherlands Pat. Nos. 0743987 and 0851867, Spain Pat. Nos. 2197193 and 2173310, Sweden Pat. Nos. 0743987 and 0851867, Switzerland Pat. Nos. 0743987 and 0851867, United Kingdom Pat. Nos. 0743987 and 0851867, U.S. Pat. Nos. 5,654,419, 5,688,648, 5,869,255, 6,177,247, 5,707,804, 6,028,190, 6,544,744, 7,015,000 and 5,728,528 and other pending and foreign patent applications.

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